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ABSTRACT

This manual provides numerous experiments for college-level microbiology students illustrating the basic principles in phage, tissue culture, and animal virology. (CS)

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# A LABORATORY MANUAL FOR INTRODUCTORY VIROLOGY
# BASED UPON DEVELOPMENTAL STUDIES WITH
# BACTERIAL AND ANIMAL VIRUSES.

by

CONNIE WONG, B. A.

Final Technical report for the National Science
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Title: "Virology as an Undergraduate
Introduction to Modern Concepts
in Biology."

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# A LABORATORY MANUAL FOR INTRODUCTORY VIROLOGY BASED UPON DEVELOPMENTAL STUDIES WITH BACTERIAL AND ANIMAL VIRUSES.

by

CONNIE WONG, B. A.

THESIS

Presented to the Faculty of the Graduate School of
The University of Texas at Austin
in Partial Fulfillment
of the Requirements
for the Degree of

MASTER OF ARTS

THE UNIVERSITY OF TEXAS AT AUSTIN
December, 1974

# PREFACE

Virus research encompasses many aspects of the virus-host relationship. One may consider important contributions from viral replication and production of progeny, the influences on host cell physiology and metabolism, the structural composition of viral particles, genetic control mechanisms and nutritional requirements of both the host cell and replicating virus particle. It is the purpose of this paper to introduce a laboratory manual with experiments in the areas of cell culture, bacterial and animal virology and to evaluate its use in undergraduate student virology laboratories.

The basic form of this manual consists of an introduction of the early studies using the techniques of the particular experiment, a description of the method employed, and more recent modifications and applications of the experimental technique to other areas. In addition, illustrations in the form of photographs, data charts, and graphs have been included to allow students to become familiar with experimental observations and results and to provide some criteria with which the student can evaluate his own findings.

Many of the experimental studies presented emphasize basic concepts in phage virology which have been established and clearly defined by early workers. These involve different aspects of the virus-host interaction from infection and adsorption, serum neutralization, viral growth and host cell metabolism to viral integration and genetic control. Studies were also extended to determining and comparing the effects of varying experimental conditions on the course of these different reactions.

At present, increasing importance and developments have been projected into the area of animal cell culture and virology. Many of the animal viruses are associated intimately with disease and the biological welfare of man and animals. The significance of these studies lies in the fact that both in vitro and in vivo systems exist and can be applied to investigation of these viruses. Most students have only been given descriptions of cell culture studies, but have little experience in handling such work in practice, e.g., preparation of an avian cell culture and its use for animal virus titrations. Such procedures have been presented to allow the students to become familiar with the principles and manipulations of animal cell work. Most cell cultures require rich serum-containing media and a $CO_2$ atmosphere. The introduction of serum-free media studies have intitated the possibility that these may prove as satisfactory substitutes for serum-containing media in student lab cultures and eventually eliminate use of complex media and special $CO_2$ incubator in routine cell growth and maintenance.

The in vivo animal studies demonstrate viral infectivity in a natural system. The study presented here of the Sindbis-mouse system enables students to observe the age-dependent resistance phenomenon in newborn and young adult mice. Such animal projects prove valuable, but limited as a routine lab exercise. Much preparation and time are necessarily required as well as continual supervision in procedures and methods.

The materials presented here have been subject to personal experimentation and evaluation as well as to student investigations. Most of the phage experiments have been employed successfully for several semesters. The cell culture and animal studies are recommended for smaller supervised groups of students.

It is hoped that this manual will enable students to acquire laboratory experience and technical skills and to apply these to the understanding of some of the basic principles in phage, tissue culture and animal virology.

# ACKNOWLEDGMENTS

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To my parents, Mr. and Mrs. Hong C. Wong, I am forever grateful for their encouragement, understanding, and support, and to them I sincerely dedicate this thesis.

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Submitted 6 December, 1974

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# EXPERIMENTS

# Introduction, Material, and Methods

# PHAGE ASSAY

Although several different methods have been devised for the determination or assay of the relative number of bacterial viruses in a specific sample, the most convenient and satisfactory method is the use of the plaque count technique which determines the number of *plaque-forming units* (*PFU*) or infectious units. A small number of phage particles and a large number of sensitive bacteria are mixed and spread on the surface of an agar plate. It is essential that the bacteria are in great excess of the phage particles in this technique. During incubation, the infectious phage particles initiate a bacteriolytic cycle at the expense of the neighboring cells. After several hours, the bacteria have grown into a dense film or lawn except in those areas where multiplying phage particles have lysed the bacteria, yielding more or less clear circular holes or *plaques* which are easily detected in the uniform bacterial lawn of growth. The plaques are, in effect, virus colonies. As with bacterial plate counts, it is assumed that each plaque originated from one infectious phage particle. The plaques may be counted, and the original titer of the suspension of infectious phage particles may then be calculated by multiplying the number of plaques by the dilution factors.

If the approximate titer of a virus suspension is not known, several different dilutions of the phage suspension must be prepared and plated to be certain that one of the plated dilutions will contain a reasonable number of plaques. If too many plaques are present, many of them will overlap, and the count will be unreliable. Since different phages form plaques of different size, no general rule can be given for the maximal number of plaques that can be counted on one plate. A general rule of thumb has been to keep the minimum over 30 PFU. Sampling error can be reduced by plating duplicate samples of each dilution and taking the average.

It is emphasized that the phage assay method is not an absolute measure of particle number. Not only are some phage particles incapable of initiating the lytic cycle, but a variety of environmental factors such as incubation temperature, medium pH, presence or absence of certain salts, as well as the type of bacterial strain, can modify the number of plaques which develop.

Two variations of the plaque assay method are the spreading method and the soft agar method. For the spreading method, 0.1 ml of the phage dilution and 0.1 ml of a suitable indicator organism are placed on the surface of an agar plate and spread over the entire surface with a sterile L-shaped glass spreader. It is also possible to prepare 10-fold dilutions of the phage using 0.9 ml of the indicator organisms in broth as the diluent, then pipetting 0.1 ml of the phage-bacteria mixture onto the surface of an agar plate and spreading. The agar surface should be dry before use, otherwise the phage will grow in the excess fluid medium and form large, confluent lytic areas instead of discrete plaques. It may be necessary to dry the surface of an inoculated agar plate in an inverted position for 15 to 20 minutes in the 37 C incubator.

2.0 ml of 24 hr broth culture of E. coli β (about 2-5 × $10^9$ cells/ml)

| | |
|---|---|
| sterile 1.0 ml pipettes (graduated in 0.1) | 7 ea |
| plates Penassay agar | 6 ea |

L-shaped glass spreading rods, 95% ethanol and water in beakers.

Procedure:

1. Pipette 0.9 ml of a young broth culture of E. coli β into each of the first three sterile Kolmer tubes in the dilution series. Into the last two Kolmer tubes put 0.9 ml of 24 hr E. coli culture.
2. Using a sterile 1.0 ml pipette, transfer 0.1 ml of the T phage suspension to the first tube and mix ($10^{-1}$). Transfer 0.1 of this mixture to the second tube using a fresh pipette.
3. Continue to make the 10-fold dilutions with the remaining tubes, making sure a fresh pipette is used for each dilution.
4. Upon completion of the dilutions, take a fresh pipette, and starting with the highest dilution (tube 5), pipette 0.1 ml of each phage-bacteria dilution onto the surface of Penassay agar plates for tubes 5, 4, and 3 ($10^{-5}$, $10^{-4}$, and $10^{-3}$ dilutions, respectively). The final plate dilutions will be $10^{-6}$, $10^{-5}$, and $10^{-4}$, respectively.
5. Again starting with the highest dilution, spread the inoculum over the entire agar surface with a sterile, glass spreading rod.

The soft agar overlay technique for assaying phages was first described by Gratia in 1936 and since has been modified by various workers (Adams, 1959). In general, the host indicator organism and phage are inoculated and mixed in a small volume (2.5 ml) of melted, cooled (43-45 C) 0.5% soft agar medium and then poured and evenly layered on the surface of a 1.5% agar base plate. The soft agar is allowed to solidify on a level surface before incubation in the upright position. The bacteria, receiving nutrients primarily from the agar base medium, yield confluent growth and form a dense lawn in the soft agar except where phage particles have replicated and formed a plaque.

A. Spreading Technique for Determination of Plaque Forming Units.

Materials: PER STUDENT

| | |
|---|---|
| sterile Kolmer tubes (3" tubes) | 5 ea |

0.1 ml of assigned phage suspension (Diluted to contain 1 to $5 \times 10^7$ PFU/ml)

8.0 ml of young broth culture of *E. coli* β (about $5.0 \times 10^7$ cells/ml)

6. Allow the inoculum to be absorbed completely by the agar before inverting and incubating the plates at 37 C for 15 to 18 hours or until the plaques are well developed and can be counted easily within the bacterial lawn.
7. Count the number of plaques for each dilution wherever possible and determine the number of PFUs in the original phage stock suspension.

B. Soft Agar Overlay Technique for Determination of PFUs.

Materials: PER STUDENT

| | |
|---|---|
| Phage suspension (diluted to contain about 1-5 × $10^7$ PFU/ml) (0.5ml) use same $\phi$ suspension as in part A. | |
| 1.0 ml *E. coli* β (about 2-5 × $10^9$ cells/ml). Remainder of 24 hr culture from part A is to be used as this indicator organism | |
| tubes penassay soft agar (45 C water bath) | 6 ea |
| plates penassay base agar | 6 ea |
| 4.5 ml dilution blanks (chilled ) (1:4 ratio of PA broth to saline) | 6 ea |
| sterile 1.0 ml graduated pipettes (graduated in 0.1) | |
| pasteur pipettes sterile | |

Procedure:

Use the same phage in this exercise that was used with the spreading method.

1. Pipette 0.5 ml of the phage suspension into a 4.5 ml dilution blank ($10^{-1}$). Mix well and transfer 0.5 ml of this dilution to a second blank and so on up to dilutions of $10^{-6}$.
2. Obtain two tubes of penassay soft agar from the 45 C water bath and using a sterile pasteur pipette add 3 drops of 24 hr culture of E. coli β into each tube of melted agar medium. With a fresh pipette, transfer 0.1 ml from the $10^{-6}$ dilution to each tube of the seeded soft agar. (Final plate dilution will be $10^{-7}$.) Mix without causing bubbles and pour the contents of each tube on the surface of separate penassay agar base plates. (Overlay by gently tilting the plate back and forth as demonstrated by the laboratory instructor.)

   NOTE: It is always advisable to pipette the indicator organism into the soft agar medium before adding the phage inoculum so as to prevent possible contamination of the indicator organism with phage.
3. Repeat procedure 2 using the $10^{-5}$ and $10^{-4}$ phage dilutions in place of $10^{-6}$ dilution. (Final plate dilutions will be $10^{-7}$, $10^{-6}$, $10^{-5}$.)
4. Allow the agar to harden before incubating the plates in the upright position in the 37 C incubator.
5. After 15 to 18 hours, count and average the number of PFUs for each dilution. Determine the titer of the original phage suspension. (Multiply the number of plaques on plate by the reciprocal of the final dilution of plate.)

6. Describe the plaque morphology of the phage, including the presence or absence of any evident halo. Also, describe the plaque morphology of other coliphages available on demonstration or from plates of other students. (Observe Figs. 1-3 for characteristic plaque morphology of some T-phages).
7. Record all results on the data sheet following the exercise.

Questions:

1. Does the plaque morphology of your phage appear homogeneous? What might be expected if one or two plaques on the agar plates appeared different?
2. If an exceedingly large number of phage are plated on a lawn of sensitive cells confluent lysis will occur; however, there will be a small number of bacterial colonies (less than 100) growing on such a plate. What would be a reasonable explanation for the appearance of these colonies?
3. If one or more of your dilution plates do not have any plaques in the lawn of cells, what are two possible explanations for this?
4. If some of your plates are completely clear and have no sign of plaques or cells growth what mistake in your procedure did you make? Assume that you did the pour plate method and did pour soft agar on surface of plate.
5. What possible effect would bubbles (in the soft agar overlay and on the plate) have?

Figure 1. Plaque assay of T7 on *Escherichia coli* β.

Figure 2. Plaque assay of T1 on *Escherichia coli* β.

Figure 3. Plaque assay of T2 on *Escherichia coli* β.

## Statistical Analysis of Data

Viral titers obtained by plaque counting have been compared with the number of physical particles obtained by electron micrograph counting. The agreement is very good for some phages (Luria *et al*., 1951). Plaque assay is a very accurate titration method. As the number of replicate platives increases, the mean becomes a better measure of the absolute number of infectious particles.

Two sources of error which influence the final value obtained are:

(1) Sampling error - resulting from random distribution of phage particles - personal technique.

(2) Diluting error - resulting from the error inherent in the diluting process. Luria and Darnell (1967) estimate that it is equal to the Probable Error (P.E.) of each step in the dilution series multiplied by the square root of total number of dilutions made.

Sampling error is caused by the nature of the sampling process and can never be completely eliminated. It can be minimized, however, by refinements in technique and by simple concentration on the work at hand. One way to measure it is by calculation of ones personal coefficient of variance (C.V.). *It is important to keep ones C.V. at a low level*. By carefully eyeing the miniscus in the pipette (do you allow the bottom of the miniscus to travel from the top of the marks or from the bottom of the marks on the pipette?), conscientiously mixing each dilution and in general STANDARDIZING the procedures (do you *always* blow out the last drop or never?) one can accomplish this. Since this error is random (may be positive or negative) rather than systematic (always either + or -), using a large sampling population will help to lessen the error. Therefore, at each dilution the more duplicate plates made the greater the theoretical accuracy.

Dilution error is systematic. It is caused by unavoidable errors in the calibration of pipettes, blanks, etc. Its calculation is based on the probable error of each step times the total number of steps. Probable error is defined as the value which, when added to or subtracted from the mean provides a range containing fifty percent of the values. The smaller this range the more accurate the data. The P.E. is used as a way of expressing accuracy of data, within its limits. (What are they? Consider the differences between relative accuracy and absolute accuracy.)

In part I, the accuracy of 1.0 ml pipettes and 0.1 ml pipettes are compared.

In part II different methods for arriving at the same final dilution are compared.

Using the data from parts I and II, it will be possible to manipulate the number in such a way as to obtain some idea of the accuracy involved.

Calculations for Part I

1. Calculate the mean (average of the 0.1 ml pipette samples and the 1.0 ml pipette samples.

$$\text{mean } (M) = \frac{\Sigma \text{ of observed}}{n}$$

2. Calculate the deviation (d) from the mean. d = difference between single observation and mean.
3. Calculate $d^2$.
4. Calculate experimental standard deviation (s).

$$s = \sqrt{\frac{\Sigma d^2}{n-1}}$$

5. Calculate coefficient of variance (C.V.).

$$\% \text{ C.V.} = \frac{s \times 100}{\text{mean}}$$

Calculations for Part II:

1. Calculate M, s, d, $d^2$ as before.
2. Calculate % P.E.

$$\% \text{ P.E.} = .67 \sqrt{\frac{\Sigma d^2}{(n-1)^2}} \times 100$$

3. Compare the results from the three methods. This may be done using P.E. - the difference between the probable error of the two results (P.E. difference) is a number which can be used to determine if the data from the two experiments are significantly different.

$$\text{P.E. difference} = \sqrt{\text{P.E.}_A^2 + \text{P.E.}_B^2}$$

The results of the two experiments can be considered significantly different if the difference between the mean of experiment A and the mean of experiment B is larger than 3 times the P. E. difference.

$$|M_A - M_B| > 3\text{P.E. diff}$$

Compare the 3 methods for significant differences.

$$|M_2 - M_4| > 3\sqrt{\text{P.E.}_2^2 + \text{P.E.}_4^2}$$

$$|M_4 - M_{13}| > 3\sqrt{\text{P.E.}_4^2 + \text{P.E.}_{13}^2}$$

$$|M_2 - M_{13}| > 3\sqrt{\text{P.E.}_2^2 + \text{P.E.}_{13}^2}$$

Experimental:

Part I

Materials: (PER STUDENT)

| | |
|---|---|
| dilute phage suspension | |
| sterile plates | 20 ea |
| Tryptose-phosphate soft agar tubes | 20 ea |
| 0.1 ml pipettes | 5 ea |
| 1.0 ml pipettes | 5 ea |

1. Using a sterile pasteur pipette put 8 drops of 24 hr E. coli β into each of 2 T-P tubes.
2. Add 0.1 ml (use a 1.0 ml pipette) of the phage to the tube and mix thoroughly. Avoid bubbles. Pour the entire contents onto the sterile plate. Swirl the plate quickly but gently so that the agar completely covers the bottom and is completely mixed. This is a "pour plate." Since the phage and bacteria are mixed all through the agar, some subsurface plaques will develop. Be sure to look on the sides, bottom and top for plaques. Use the same pipette for

both tubes.

3. Repeat steps 1 and 2 until 10 plates have been poured.
4. Use a 0.1 ml pipette and repeat above 1, 2, and 3.
5. Count plaques at 15-18 hrs and record on charts provided.

## Part II

**Materials:** (PER STUDENT)

| | |
|---|---|
| phage suspension | |
| sterile plates | 15 ea |
| T-P agar tubes | 15 ea |
| 9.9 ml dilution blanks | 2 ea |
| 4.5 ml dilution blanks | 4 ea |
| 1.0 ml dilution blanks | 13 ea |
| 0.1 ml pipettes | 11 ea |
| 1.0 ml pipettes | 17 ea |

**Methods:**

1. Prepare 3 dilution series with 2, 4 and 13 steps, the final dilution being approximately the same.

| Dilution Factor Of Each Step | Sample Size | Volume | No. Steps | Final Dilution Factor |
|---|---|---|---|---|
| (a) 1:100 | 1% | 0.1 ml/9.9 ml | 2 | $1 \times 10^{-4}$ |
| (b) 1:10 | 10% | 0.5 ml/4.5 ml | 4 | $1 \times 10^{-4}$ |
| (c) 1:2 | 50% | 1.0 ml/1.0 ml | 13 | $1.2 \times 10^{-4}$ |

For each dilution series (a, b, c) make 5 pour plates from the last tube (highest dilution). Use 0.5 ml/tube. REMEMBER THIS WHEN MAKING CALCULATIONS!

DATA TABLE PART I:

| 0.1 ml pipette | | | | 1.0 ml pipette | | | |
|---|---|---|---|---|---|---|---|
| Plate | Plate Count PFU/plate | Deviation From Mean d | Deviation 2 $d^2$ | Plate | Plate Count PFU/plate | Deviation From Mean d | Deviation 2 $d^2$ |
| 1 | | | | 1 | | | |
| 2 | | | | 2 | | | |
| 3 | | | | 3 | | | |
| 4 | | | | 4 | | | |
| 5 | | | | 5 | | | |
| 6 | | | | 6 | | | |
| 7 | | | | 7 | | | |
| 8 | | | | 8 | | | |
| 9 | | | | 9 | | | |
| 10 | | | | 10 | | | |
| Sum(Σ) | | | | Sum(Σ) | | | |

DATA TABLE PART II:

| 2 Step Series | | | | 4 Step Series | | | | 13 Step Series | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate | Plate Count | d | $d^2$ | Plate | Plate Count | d | $d^2$ | Plate | Plate Count | d | $d^2$ |
| 1 | | | | 1 | | | | 1 | | | |
| 2 | | | | 2 | | | | 2 | | | |
| 3 | | | | 3 | | | | 3 | | | |
| 4 | | | | 4 | | | | 4 | | | |
| 5 | | | | 5 | | | | 5 | | | |
| Sum(Σ) | | | | Sum(Σ) | | | | Sum(Σ) | | | |
| Mean = | | | | Mean = | | | | Mean = | | | |
| S = | | | | S = | | | | S = | | | |
| P.E. = | | | | P.E. = | | | | P.E. = | | | |

## UNRESTRICTED CELL DIVISION

Under ideal conditions a bacterial cell such as *Escherichia coli* can divide into two individual cells every fifteen to twenty minutes (1). This continued doubling is usually expressed as a geometric progression (5): $2^0$, $2^1$, $2^2$, $2^3$. . . . $2^n$ and proceeds as a function of time (7). Ideally, rates of multiplication and changes in a bacterial population can be represented schematically as a growth curve consisting of various phases of growth, as originally proposed by Buchana (2) and later by Monod (4). Such a curve was constructed by enumeration from "viable counts" as obtained from conventional spread plate assays of designated samples from a young, growing bacterial culture, and the results plotted as a function of time. However, an apparent discrepancy does exist between the lower viable counts when compared to total cell determination, as depicted by microscopic counts or optical density measurements of turbidity of the culture (11). The number of cells obtained in the total plate count does not correspond to the absolute number of microbial cells present in a culture because of the following inherent sources of error in the dilution plate counting technique: only living cells develop into colonies; clumps (as in many *Staphylococcus* sp.) and chains of cells (e.g. many *Bacillus* sp.) develop as single colonies and colonies develop only from those cells for which the plating conditions are suitable to support growth.

The experimental studies on bacterial growth and metabolism have been most abundant in the early literature. Extensive research and descriptions have been reviewed by many workers in the past, who have examined the different phases of the growth curve and effects of the many variables on the system, as well as any modifications of these variables on bacterial growth. These variables include cell characteristics such as specific nutritional requirements, and the synthesis of cofactors, vitamins and metal chelating compounds (e.g. $Fe^{+3}$ chelating compounds) etc. Growth also depends on variables of the culture conditions such as salt and ion concentrations, nature and concentrations of growth media, temperature, pH, and aeration. Some studies have also investigated changes in the structural nature of growing cells, thereby introducing yet another variable.

,Today, more elaborate experimental investigations are being conducted in an effort to examine effects of these specific variables in relation to different cellular activities and functions in a bacterial population.

Required reading for this lab is reference 11, pp. 298-318.

## UNRESTRICTED CELL DIVISION

Materials: PER PAIR

| | | |
|---|---|---|
| 7" tube with 5.0 ml of 24 hr E. coli β culture in Penassay broth | | 1 ea |
| 500 ml flask with 100 ml of Penassay broth --prewarmed to 37 C | | 1 ea |
| NA plates | | 48 ea |
| 9.9 ml dilution blank | dilution fluid is 1:4 ratio of PA broth to saline | 12 ea |
| 4.5 ml dilution blank | | 21 ea |
| cans of 1 ml pipettes (50) | | 2 ea |
| 10 ml pipettes | | 8 ea |
| Spectronic 20 with prepared blank containing 3.0 ml of sterile broth the cells are grown in (NOTE: must use the same machine throughout the entire experiment) | | 1 ea |
| clean cuvette | | 1 ea |
| 95% EtOH and L-rods | | |

Method:

1. Preparation of assay of 24 hr *E. coli* β culture.

   (a) Take O.D. of 24 hr culture by pipetting 3.0 ml of 24 hr culture into a clean spect. 20 cuvette (using 10 ml pipette). Read the absorbance as O.D. (optical density).

   (b) Make $10^{-2}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$ dilutions of 24 hr *E. coli* β in that order, using only two 9.9 ml and three 4.5 ml blanks.

   (c) Using the spread plate method with flamed glass L-rod, prepare 2 plates of each of the following final plate dilutions $10^{-6}$, $10^{-7}$, $10^{-8}$. Surface spread plates are prepared by pipetting only 0.1 ml of the dilution onto surface of agar. Remember that plating 0.1 ml of a dilution is equivalent to making an additional $10^{-1}$ dilution. All dilution plates should be dried before use to remove excess surface moisture on agar which permits spreading colonies to develop ("spreaders"). Invert plates on clean shelf in 37 C incubator, remove bottoms and allow them to rest on the edge of the tops at a slight angle for 30 min. Do not dry any longer and remember to keep plates closed during plating and when incubating for growth. It would save you time if each student makes sure that he has 9 plates drying in such a manner 30 minutes before class.

   (d) Spread 0.1 ml inoculum evenly and allow to dry completely. Invert plates and incubate at 37 C for 18 hrs.

2. Preparations for growth curve experiment at 0, 30, 60, 90, 120, 150, and 180 minutes.

(a) Inoculation of fresh medium. At 0 min transfer 3.0 ml of 24 hr E. coli β to 100 ml of prewarmed broth in 500 ml flask and mix well.

(b) Assay of inoculated flask. At 0 min pipette 0.1 ml of inoculated broth into 9.9 ml dilution blank. This $10^{-2}$ dilution is the first dilution in the series described in (c). Then using a 10 ml pipette, take out 3.0 ml from the same flask and put into clean cuvette. Put flask in 37 C shaker immediately. Take O.D. of volume in cuvette immediately, and the other partner should continue to make the dilutions platings described in (c).

(c) Complete the appropriate dilution $10^{-3}$, $10^{-4}$, $10^{-5}$[these are dilutions of flask culture] using only 4.5 ml blanks. Using the spread plate method prepare 2 plates of each of the following final plate dilutions: $10^{-4}$, $10^{-5}$, $10^{-6}$.

(d) Follow the same directions in (b) and (c) for 30, 60, 90 minutes.

(e) For time intervals 120, 150, 180 minutes follow step (b) but the tube dilutions will be $10^{-2}$, $10^{-4}$, $10^{-5}$, $10^{-6}$. Spread plates prepared in duplicate for the following final plate dilutions $10^{-5}$, $10^{-6}$, $10^{-7}$.

3. All plates needed to be labeled on the bottoms only with the following information: time interval (e.g., 0, 30 min etc.); final plate dilutions; and your initials. Incubate plates for 15-18 hrs.

Data will include Table (see below) with the following information for each of the seven time intervals and the 24 hr culture: all plate counts, the titer (CFU/ml); and O.D.

| Time (Min.) | | | Final Plate Dilutions | | | Titer (CFU/ml) | O.D. |
|---|---|---|---|---|---|---|---|
| | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | | |
| 0 | | | | | | | |
| 30 | | | | | | | |
| . | | | | | | | |
| . | | | | | | | |
| . | | | | | | | |
| . | | | | | | | |
| . | | | | | | | |
| 24 hrs | | | | | | | |

Also included in the Data section will be the following three graphs: (1) on 304 cycle semilog paper plot CFU/ml on vertical axis vs. time [do not include 24 hr value]; (2) on linear graph paper plot O.D. on vertical axis vs. time [do not include 24 hr value]; and (3) on 3-4 cycle semilog paper plot CFU/ml on vertical axis and plot the corresponding O.D. values for each viable cell count on horizontal axis [include 24 hr values].

Explain briefly, but concisely, each of these graphs by describing the kind of information each graph tells you. [Hint: graphs 1 and 2 are both similar curves, but not exactly identical. Why?]

What conclusions can you draw from the two similar graphs 1 & 2 and from graph 3? These should include statements about the pattern of _E. coli_ growth and the kind of relationship between CFU/ml and O.D.

Questions

1. Using graph 1 calculate the generation time of E. coli β during the log phase under these growing conditions. Show all equations and calculations.
2. Using graph 1 calculate the length of the lag period. (See Stanier p. 309 for directions and diagram.)
3. In what growth phase is the 2.5 hr E. coli β grown under these conditions?

   In phage assay experiment a 24 hr E. coli β culture grown as you did is used as indicator, what phase of growth is it in?

## ONE STEP GROWTH OF A BACTERIOPHAGE

The quantitative study of the phage-bacterium interaction was initially done in 1926 by D'Herelle (6) who demonstrated that by assaying designated samples from an infected culture, viral growth could be observed to proceed in a stepwise fashion, resulting in the production of progeny virus. This sequence of events was quantitated by devising the "one-step growth experiment" in 1939 by Ellis and Delbruck (5).

The one-step growth experiment of Ellis and Delbruck clearly presented the general nature and kinetics of viral multiplication in a population of infected cells under suitable culture conditions. The kinetics of the multiplication is measured by an infective center assay. The infective center is the unit that forms a single plaque; one infective center could have been caused by a single unadsorbed phage or an infected cell, which may have a varying number of intracellular infectious mature progeny phages in its cytoplasm. Therefore, in this method an infected cell with no intracellular mature particles at the time of sampling is not distinguishable from an infected cell which has not lysed yet but has many mature phages in it. The basic procedure involves the infection of a young sensitive culture of cells with a designated amount of phage. The phage are allowed to absorb for 5 min; after this,the phage-host mixture is diluted in antiserum with a high titer for a period of 5 min at 37 C to neutralize all unadsorbed phage. After this step the phage-host antiserum mix is diluted 1:500 (FGT) and the FGT is diluted 1:100 (SGT). This serves two functions: (a) it dilutes out the antibodies so that none of the phages released later on are neutralized; (b) by diluting out the residual uninfected host cells they will not be infected by phages released later on. Samples of the FGT and SGT are plated at various times to determine the instantaneous number of infective units. This infective center assay is the experiment student #1 of each pair will be doing.

Other workers examined intracellular events through premature lysis of infected cells. Doermann (4) devised an experiment to assay the number of intracellular phages as they accumulated in the cytoplasm of infected cell before cell lysis. He artifically lysed the infected cells sampled concurrently from the same FGT and SGT tubes of the one-step growth experiment mentioned above. Since all cells are lysed before plating and the unadsorbed phages after 5 min were neutralized, the titer of this method represents the total number of intracellular PFU/ml (infectious phage = plaque forming unit) released by artificial lysis and the number of PFU/ml released as a result of spontaneous lysis. This is not an infective center assay because the number of plaques caused by a single infected cell will depend on the number of intracellular particles it had at the time of sampling, i.e. the stage of growth cycle the replicating phage was in. The first method developed for artificially lysing infected cells was the use of high multiplicities of infection of phage particles which initiated the process of "lysis-from-without" (7). Sometimes sonic disintegration (1) or chemical agents, e.g. cyanide (1, 4) were applied as sources of all disruption. Though different compounds have been used from time to time, the more generally useful method employs the addition of chloroform to phage-infected cultures. This assay of intracellular phage is the one student #2 of each pair will do.

The one-step growth curve (infective center assay) showed that for a period of time after infection the number of infective centers, i.e. infected cells, remains constant, this was defined as the latent period. It represents the time between the infection of the culture of cells with parental phages and the time it takes for the infected cells to release new progeny phages. As soon as the titer begins to rise the latent period ends and the rise period begins and continues until a plateau of PFU/ml is reached. The rise period is the time during which the infected cells are lysing as each one reaches the end to the viral replication cycle. As soon as all of the infected cells lyse a plateau is reached and does not increase because progeny phage and residual susceptible cells have been diluted out shortly after the initial adsorption period. The ratio of the final infective center plateau ($IC_p$) to the original infective center titer (IC) is the burst size; this represents the average number of phages released by each infected cell. Since this infective center assay is only able to detect PFU's after they were released from cell by spontaneous "lysis-from-within" little was known about the kinetics of intracellular phage maturation until Doermann's intracellular phage assay experiment. This experiment studied intracellular phage development by artificially lysing the infected cells during the latent period, thus releasing any and all infectious particles which are then detected by the assay. Doermann found a very surprising and unexpected phenomenon, there were no infectious particles released from the infected cells artificially lysed during the early part of latent period, not even the original infecting phage (parental phage). After a short time, however, intracellular PFU's begin to appear during late latent period and increased in number throughout the remainder of the latent period. The number of PFU's assayed by this method increased during rise period also due to the normal release of progeny phage by spontaneous "lysis-from-within" as well as the release of intracellular phages from infected cells that were artificially lysed-from-without by a high multiplicity of infection by phages (you will artificially lyse cell with chloroform). The PFU's assayed by this method levels off to the same plateau as obtained by the one-step growth curve. The time after infection and before any infectious phage are detectable in the cytoplasm of artifically lysed cells is called eclipse of the latent period. The time between the end of the eclipse and the time of cell "lysis-from-within" is the maturature time of the latent period, and it is characterized by the presence of infectious mature progeny phages in the cytoplasm of artificially lysed cells.

Various modifications of the one-step growth experiment have been devised to study different variables. One important extension was the "single-burst experiment" (3) which determined and compared the virus yields from individual bursts of a phage-infected bacterial suspension diluted into a series of dilutions to contain less than one infected bacterium. The spontaneously lyse, and thus, the length of the growth cycle is different for individual cells. This suggested that the burst sizes and growth cycle times obtained from a one-step growth experiment are average values of a population of infected cells. In a population of cells the external environment of each cell may be kept nearly constant, but the internal environments rarely are, e.g. physiological age and conditions. The single-burst experiments of Bentzon, Maalde, and Rausch (2) confirmed one other fact. The results obtained from Doermann's intracellular phage assay on a population of cells suggested that the increase in intracellular phage particles increased linearly with time. At the end of eclipse the number of PFU's increased at a constant rate until the end of latent period and showed linear growth kinetics. The above study on single cell intracellular infectious phage development confirmed that mature phage are formed at a linear rate not exponential. This is no reflection on how the parental and progeny nucleic acid molecules (DNA) might replicate, but it does eliminate binary fission as a mechanism of phage multiplication since it is characterized by exponential growth rate as in the case of cell division. The rate that mature phage particles are assembled is a constant linear rate.

# ONE STEP GROWTH OF A BACTERIOPHAGE

Materials: PER PAIR OF STUDENTS

| | |
|---|---|
| 7" tube with 8 ml 24 hr *E. coli* β indicator | 1 ea |
| 5" tube with exactly 4.5 ml of 2.5 hr *E. coli* β | 1 ea |
| 3" tube with phage stock 1-5 × $10^7$ PFU/ml | 1 ea |
| 5" tube with 1.8 ml of phage antiserum diluted 1:20 | 1 ea |
| sterile petri dishes | 48 |
| tryptose-phosphate soft agar tubes with 7.5 ml | 48 |
| 4.0 ml dilution blank in 5" tube | 1 ea |
| 4.5 ml dilution blank with 1.0 ml $CHCl_3$ in 5" tube | 12 ea |
| 9.0 ml dilution blank in 7" tube | 1 ea |
| 18.0 ml dilution blank (in 50 ml flasks) | 1 ea |
| [dilution fluid is 1:4 ratio of PA broth to saline] | |
| 19.8 ml dilution blank (in 50 ml flasks) | 1 ea |
| 0.1 ml pipette | 2 ea |
| 1.0 ml pipette | 40 ea |
| pasteur pipettes | 6 ea |
| ice water bath | |
| 10 ml pipettes | 10 cans/lap |

Method:

This experiment will be done in pairs. Student #1 will be assaying the FGT and SGT tubes by the infective center assay (one-step growth curve). Student #2 will be assaying the same FGT and SGT tubes by the intracellular phage assay. Both students assist in preparing the FGT and SGT tubes (Steps 1-5):

1. Prepare and label the following materials:
   a. Student #1: 2 plates for each sampling time (15, 20, 25, 30 45, 60 min) for the FGT and 2 plates for each sampling time for SGT.
   b. Student #2 do the same as above. (Sampling times will be 10, 15, 20, 25, 30, 45 min.)
   c. Both students prepare 1 of each of the following: 4.0 ml, and 9.0 ml, 18.0 ml, and 19.8 ml dilution blanks all kept at 37 C and labeled with your initials.
   d. Student #2 label 12 chilled 4.5 ml dilution blanks with 1.0 ml of $CHCl_3$ with your initials and FGT and SGT, sampling time and keep these in ice water bath.
2. At 0 min infect 4.5 ml of E. coli β (2.5 hr) with 0.5 ml of the designated phage stock. Mix well by shaking several times. Allow 3 min for phage attachment in 37 C water bath. Record this 0 time for further reference. (1:10 dilution of phage stock)
3. Dilute the phage-host mixture made in step 2, after 3 min, 1:10 in antiserum (0.2 ml of ϕ-host mixture in 1.8 ml of antiserum of a 1:20 dilution kept at 37 C). Allow 5 min for neutralization of free phages to take place at 37 C. (1:100 dilution of phage stock)

4. Dilute the phage-host antiserum mixture made in step 3, after 5 min, in the following way using the dilutions previously kept at 37 C. Be sure to warm each pipette by passing over the flame several times before making dilutions. (The following steps must be done rapidly -- in 2 min and no more.)

   a. Transfer 1.0 ml of the phage-host-antiserum mixture into a 4.0 ml blank, put 1.0 ml of this dilution into a 9.0 ml blank, then put 2.0 ml of this dilution into a 18.0 ml blank. This represents the First Growth Tube, FGT, which is a 1:500 (or $2 \times 10^{-3}$) dilution of the phage-host-antiserum mixture made in step 3 and a 1:50,000 ($2 \times 10^{-6}$) dilution of the original phage stock.

   b. Immediately dilute the FGT 1:100 (0.2 ml into an 19.8 ml blank). This represents the Second Growth Tube, SGT, which is a 1:5,000,000 dilution of the original phage stock ($2 \times 10^{-7}$).

   NOTE: The moi in phage-host mix is almost 0.1. Assuming 2/3 of the particles attach to the cells, about $6\text{-}7 \times 10^6$ cells/ml will be infected. Dilution through antiserum (step 3) brings this to $6\text{-}7 \times 10^5$ cells/ml. Further dilution brings the expected FGT infected cell titer to $1 \times 10^3$/ml and that of SGT will be about 10 infected cells/ml.

   Maintain FGT and SGT tubes at 37 C throughout the experiment.

5. By the time step 4 is completed no more than 10 minutes have elapsed from time 0 min (step 2) so that plating of samples from FGT and SGT for student #2 will begin at exactly 10' after infection (see step #7). Sampling for student #1 will begin at 15' (see step #6).

6. Student #1 at 15 min time, with a warm sterile 1.0 ml pipette will sample 1.0 ml from the FGT and inoculate 0.5 ml into each of 2 soft agar tubes seeded with 6 drops of *E. coli* β indicator. Mix and pour into sterile petri dishes (plated undiluted FGT). Immediately repeat the procedure for the SGT (plate undiluted FGT).

7. Student #2 at 10 min time will sample from the FGT by transferring 0.5 ml to a chilled dilution blank 4.5 ml with 1.0 ml of $CHCl_3$. Shake vigorously and allow for separation of two layers in ice bath before plating (15-20 min later). Immediately repeat the same procedure for SGT.

   When layers separated, you will make 2 plates of FGT for each sampling time by inoculating each of two soft agar tubes, already seeded with 6 drops of *E. coli*, with 0.5 ml of the aqueous phase only. This is a 1:10 dilution of the FGT you have plated. Repeat the same plating procedure for the SGT dilution $CHCl_3$ tubes at each sampling time. This is a 1:10 dilution of SGT you have plated.

   NOTE: Both students should sample from the FGT and SGT at the same time to keep a fairly close time schedule. Student #1 will plate undiluted samples immediately, student #2 will not start plating the 1:10 diluted samples until after the 30 min samples have been taken.

8. At 20', 25', 30', 45' and 60' student #1 should repeat step 6 and student #2 repeat step 7 for 15, 20, 25, 30, and 45 min intervals. Incubate all plates for 15-18 hrs at 37 C.

   The data section will include the following tables:

Table 1: One-Step Growth Experiment

| | | Time of Samples | | | | | |
|---|---|---|---|---|---|---|---|
| | | 15 | 20 | 25 | 30 | 45 | 60 |
| FGT | (1) plate counts; undil. | | | | | | |
| | (2) titer (PFU/ml) | =ICo | | | | | |
| | | | | | | | |
| SGT | (1) plate counts; undil. | | | | | | |
| | (2) titer (PFU/ml) | | | | | | =ICp |

Table 2: Intracellular Phage Assay

| | | 10 | 15 | 20 | 25 | 30 | 45 |
|---|---|---|---|---|---|---|---|
| FGT | (1) plate counts of 1:10 | | | | | | |
| | (2) titer (PFU/ml) | | | | | | |
| | (sum of plates × 10) | | | | | | |
| | | | | | | | |
| SGT | (1) plate counts of 1:10 | | | | | | |
| | (2) titer (PFU/ml) | | | | | | |
| | (sum of plates × 10) | | | | | | |

The data section will also include the following graph. On one 3 or 4 cycle semilog paper plot both sets of data as PFU/ml vs time and clearly label which line represents which curve. Also label the following items: latent period, eclipse, maturation, rise period and burst size. Explain in detail what the graph shows and how and why the two curves are shaped differently.

Questions:

1. Burst size = $\frac{ICp}{ICo}$ ; see paragraph 4. Calculate the burst size from your graph and data of the one-step growth experiment.
2. What is the length of the latent period?
3. At what time after infection did the first mature progeny appear in the cytoplasm of infected cells? In the external environment of

the cells? Answer in terms of minutes after infection and name of the different phase you have labeled on your graph.

4. What % of phage attachment took place in 3 min at 37 C?

The titer of undiluted phage stock =

The titer of phage in host mixture =

The titer of infected cells in phage-host mixture = # of absorbed $\phi$ = (see step 4 in procedure).

Fraction of adsorbed phage × 100 = % phage attached.

## ADSORPTION OF BACTERIOPHAGE TO HOMOLOGOUS CELLS

It was demonstrated by Krueger (1931) and Schlesinger (1932) that the logarithm of the ratio of unadsorbed phage to the original phage input (i.e. fraction of phages remaining unadsorbed, P/Po) decreases with time and is porportional to cell concentration. The rate of velocity of adsorption (k) is directly proportional to cell concentration. This suggested that the kinetics of phage adsorption is that of first-order reactions.

Besides virus and cell concentrations, the adsorption rate is also a function of temperature, medium viscosity, and the physiological state or age of the bacteria. Under constant environmental conditions and uniform culture conditions of the host cells, the kinetics of phage adsorption can be expressed by equation 1:

Equation 1 $$-\frac{dP}{dt} = k(B)(P);$$

Integration of equation 1 yields:

Equation 2 $$\log \frac{P}{Po} = -\frac{1}{2.3} k(B)(t);$$

to solve for k:

Equation 3 $$k = \frac{2.3}{(B)t} \log \frac{Po}{Pt}$$

In this experiment you will measure the adsorption rate constant. In equation 3 Po and B are measured directly before the experiment by assaying the phage and 2.5 hr cell suspension. The value of P changes with time and can be measured experimentally by two different methods:

1. Assay directly for unadsorbed phage
2. Assay indirectly by determining the number of infected bacteria.

For both measurements the samples will be taken out of the same phage-host mixture but the treatment will be different depending on which of the above methods is used. In method (1) the objective is to measure all the infectious phage particles that have not adsorbed to a cell. Each cell can attach more than one phage, but only one phage will enter into a productive replication cycle and the other phages on the wall become inactivated. To insure accurate results not more than one phage can attach to any one cell; therefore, a low multiplicity of infection (less than one) is used in the original host-phage mixture. The original ratio of phage to cell is expressed as multiplicity of infection (m.o.i.). Before the number of free phages can be measured at each time interval, the previously infected bacteria in the samples must be artificially lysed in order to prevent them from releasing more mature particles later on when they are on the plate. Chloroform ($CHCl_3$) causes the lysis of

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$P_o$ = phage titer at time 0 PFU/ml
P = titer of unadsorbed phage at t minutes
B = cell concentration as CFU/ml
t = minutes
k = adsorption rate constant with dimensions ml/min

cells immediately. During the first 15 minutes after infection the phage is in the eclipse of the latent period, and there are no infectious phages in the cytoplasm of the cell during this time.

In method (2) the measurement of unadsorbed phage (P) is indirect. If the moi is low, then the number of infected cells can be equated to the number of adsorbed phage. The value of unadsorbed phage will be:

Equation 4 $$P = P_o - (\text{number of infected bacteria at time } t)$$

The first step at each time is to dilute the phage-host mixture in Anti-T2 serum; this inactivates all unadsorbed phage particles so that they can no longer infect cells. This treatment does not affect the cells that have become infected while in the phage-host mixture. This dilution in antiserum must be incubated at 37 C for 5 min to allow sufficient time for a phage to become inactivated but it can not be allowed to incubate for more than 5 min. If it is incubated longer than the originally infected cells, it will burst in this tube and the released particles may become inactivated by any excess anti-T2 antibodies. You want to be sure to plate these samples before the time of burst because this method is measuring the number of infective centers which is equal to the number of infected cells. (The original infected cell results in one plaque in which T was the central starting point, thus it is called the infective center, it is analogous to the single cell that results in one colony.)

## ADSORPTION OF BACTERIOPHAGE TO HOMOLOGOUS CELLS

**Materials**:

| | | |
|---|---|---|
| 24 hr *E. coli* β in 5" tube (indicator) | 7.0 | ml |
| 2.5 hr *E. coli* β in *50 ml flask* | 30 | ml |
| T2 stock | 3 | ml |
| tubes with 1.8 ml of anti-T2 serum | 4 | ea |
| tubes of Tryptose-Phosphate agar | 56 | ea |
| plates | 56 | ea |
| 9.9 ml dilution blank | 4 | ea |
| 0.9 ml dilution blank | 28 | ea |
| 4.5 ml dilution blank | 8 | ea |
| 1.8 ml dilution blank | 4 | ea |
| chloroform | 4 | ml |
| 4 cans of 1.0 ml pipettes | | |
| 10 ml pipette | 1 | ea |
| ice and ice bucket | | |

You will be working in groups of 4 on this experiment. Pair #1 will be assaying the unadsorbed phage by Method #1; similarly, pair #2 will be doing Method #2. The results each pair gets are to be compared and included in each individual's lab report. Each group of 4 will have a 2.5 hr *E. coli* culture and a phage stock, which are to be used by both pairs in the group.

Method:

1. Each pair assay your 2.5 hr E. coli β culture just prior to doing experiment by the pour plate method.
2. Each pair assay your phage stock by pour plate method. Remember to put indicator organism in soft agar tube before adding 0.5 ml of phage dilution.
3. Each group of 4 at zero time add 2 ml of undiluted phage to 18 ml of 2.5 hr E. coli β (this is a 1:10 dilution of original phage stock). Mix by swirling vigorously then incubate at 37 C. This phage-host mixture will be used by Pair 1 and Pair 2 for their assays.
4. Sampling will be 2', 5', 10' and 15'.

PAIR 1 - METHOD 1: Assay of unadsorbed phage (Delbruck 1945) as follows

(a) dilute 0.2 ml of phage host mixture (1:10 dilution) into cold solution of 1.0 ml $ChCl_3$ and 1.8 ml of dilution fluid. Shake vigorously; let stand in ice water until the two phases have separated.

(b) dilute from the upper aqueous phase (must be clear) using three 0.9 ml and two 4.5 ml blanks only. Make duplicate plate of tube dilutions $10^{-3}$, $10^{-4}$, $10^{-5}$. Remember to add indicator to soft agar tubes before adding 0.5 ml of phage dilution. When calculating dilution factors and titer, remember to include the $10^{-1}$ dilution step (a).

(c) Repeat step (b) at each sampling time, and be sure to keep on the sampling time table exactly because infected cells after 15 min will contain mature phage particles in cytoplasm which will be released after $CHCl_3$ treatment.

PAIR 2 - METHOD 2: Determination of numbers of infected cells (Fredricq 1952).

(a) dilute the phage-host mixture $10^{-1}$, 0.2 ml into 1.8 ml of prewarmed anti-T2 serum (37 C). Use serum dilution as distributed.

(b) Incubate 37 C for 5 min only, then dilute further using only three 4.5 ml blanks. Make duplicate plate of the $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ *tube* dilutions, remembering to add indicator before adding 0.5 ml of phage dilutions to soft agar.

(c) Repeat step (b) at each time interval. This method requires that the timing be perfect, so to keep from getting behind both partners should work on this when necessary. Both pairs should each have a complete set of data for both methods. Include the following data tables:

TABLE 1

| Stocks | Plate Counts<br>$10^{-6}$ | <br>$10^{-7}$ | Original Titer Of Stock | Titer in Phage/ Host Mixture |
|---|---|---|---|---|
| $\phi$ | | | | = Po |
| 2.5 hr *E. coli* | | | | = B |

METHOD 1

| Time | $10^{-2}$ | PFU/plate<br>$10^{-3}$ | $10^{-4}$ | $10^{-5}$ | Titer of Unadsorbed Phage (P) |
|---|---|---|---|---|---|
| 2 | | | | | |
| . | | | | | |
| . | | | | | |
| . | | | | | |
| 15 | | | | | |

METHOD 2

| Time | PFU/plate<br>$10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | Titer of Infected Cells | Titer of Unadsorbed Phage (Equation 4) |
|---|---|---|---|---|---|---|
| 2 | | | | | | |
| . | | | | | | |
| . | | | | | | |
| . | | | | | | |
| 15 | | | | | | |

TABLE 4

| Time | k(Method 1) | k'(Method 2) | # of unadsorbed ϕ | # inf. + cells |
|---|---|---|---|---|
| 2 | | | | |
| . | | | | |
| . | | | | |
| . | | | | |
| 15 | | | | |

The data section should also include the following graph; plotting 2 curves on the same set of axis of 3 or 4 cycle semi-log paper. Let the verticle axis represent the titers, in case of one curve the titer will be of unadsorbed phage from Method 1, and for the other curve titer will be of infected cells from Method 2. The horizontal axis is time. Explain briefly the information shown in the graph, also explain any unexpected or unusual aberrations in the data if there are any (easily seen in graph as "strange" looking curves). What can you conclude about the value of the adsorption rate constant in this phage-host system, and the effect of increasing time on its value?

Questions:

1. Does the number of unadsorbed phage plus the number of infected

cells add up to the same number at each time? Explain briefly.

2. Anti-T2 serum inactivates unadsorbed T2 particles, i.e., they are no longer infectious. Antiserum contains antibodies directed against specific and nonspecific proteins. What protein(s) of T2 must these antibodies be directed against in order to inactivate the T2 particle?

3. Calculate the moi in your original phage-host mixture.

$$\text{moi} = \frac{\text{total \# phages added}}{\text{total cells present}} = \frac{\text{original phage titer (PFU/ml)} \times 2.0\text{ ml}}{\text{original cell titer (CFU/ml)} \times 18.0\text{ ml}}$$

# KINETICS OF SERUM NEUTRALIZATION

Early studies have indicated that bacteriophages can function as specific antigens (antigens are foreign proteins) to illicit the productions of specific antibodies. This was first established in 1921 by Bordet and Cuica (5) who inoculated phage lysates into rabbits and obtained sera with the ability to inactivate phage particles. This immune sera from the rabbit contains many contaminating antibodies besides the specific antibodies directed against the phage. This happens because the phage preparation used to immunize by a series of intravenous injections, contains many contaminating fragments of bacterial cell wall, the components of which are also immunogenic (i.e. capable of inducing the formation of specific antibodies directed against it, the antigen). A purified preparation of antiphage antibodies can be prepared by treating this immune sera with artificially prepared cell wall fragments of uninfected cells of the same type the phages are grown on. After these cell wall fragments have precipitated out the antibacterial antibodies just the antiphage antibodies are left.

One outstanding characteristic of immune phage sera is that it results in the inactivation or neutralization of infectious phage particles when the specific antibody contacts the phage. Thus the mixing of immune sera with the homologous phage is termed serum neutralization. Several different antibodies can be induced by a phage particle, because each different protein on the phage surface will induce the production of a specific antibody directed against it. Some of the antibodies directed against a phage particle can not neutralize it; however, the antibodies directed against a certain "critical site" are able to neutralize the phage. For T2, the neutralizing antibodies are directed against the tail proteins, the structures involved in recognition and attachment. The neutralizing antibodies must interfere with an early step in phage infection because once the phage genetic material has entered the cell the antibodies have no effect on phage replication, maturation or release. The neutralizing effect may be due to interference with normal absorption and/or the injection of phage genome into cell. The former seems to be the most likely explanation for the T-even phages. However, the later mechanism probably occurs with other phages, since it has been reported that neutralized phages of some types can still attach to sensitive cells (6).

Work on serum neutralization has been abundant in the literature, with extensive investigations by different workers for various phage systems, e.g. coliphages (8, 13-17), small RNA phages of $MS_2$, $f_2$, WB (10, 12, 21), and small DNA phages of $\phi$X174 (11, 18, 22, 25). Early studies also evaluated important effects of certain variables on neutralization-temperature (7), virus concentration, antibody concentration, pH, and various ionic requirements (15). These variables will be kept constant in your experiment.

It was recognized that immune phage antiserum possessed a great degree of phage specificity; in other words, anti-T2 serum will have the greatest neutralizing ability against T2 and will have no neutralizing effect on unrelated phages. This is because unrelated phages will have unrelated neutralizing antigens. (The phage proteins that induce neutralizing antibodies are called neutralizing antigens.) In cases of closely related phages the antisera directed against one phage type will not only neutralize the homologous phage (the phage that the antiserum is specifically directed against ) but will also neutralize other closely related phages, though less efficiently. This phenomenon is called crossreaction because the same antibody can "crossreact" with two different antigens. In other words, the same neutralizing antibody is crossreacting with two different but closely related phages. These two phages are then said to be serologically related. Serological crossreaction provided the first method of establishing groups of closely related bacteriophages, long before morphology, host range and genetic criteria were even known (6).

Early observations also indicated that fresh normal serum from different non-immunized animals demonstrated some degree of neutralizing activity against several T-phages. Such activity has been proposed to be attributable to several possible mechanisms, e.g., the presence of low levels of normal phage-neutralizing antibodies (16, 19, 24, 25) and/or certain serum factors such as properidin and complement (4, 9, 27), and/or heat inactivation due to incubation at 37 C during reaction. A recent report (2) has demonstrated that phage neutralization can be amplified by diverse serum agents, e.g., complement, heterologous antiglobulin, or anti-allotypic serum.

The kinetics studies of neutralization began with the work of Prausnitz (20) who observed that the reaction between phage and antibody occurred as a relatively slow porcess, and that a small portion of the phage population appeared resistant to neutralization activity. Little work had been done since, until the kinetic studies of Andrewes and

Elford (3). They investigated neutralization at varying phage and antibody concentrations, observing that such a reaction proceeds at a measurable rate [Kn] as a function of time. Their experimental work introduced an important phenomenon known as the "percentage law," stating that "over a very wide range a given dilution serum neutralized in a given time an approximately constant percentage of phage, however much phage there was present." This percentage of phage is between 90 and 99% (3, 28).

To measure the rate and efficiency of serum neutralization, the neutralization rate constant [Kn] is measured experimentally. The larger the value of Kn, the greater the neutralizing potency of the particular antiserum used. A mix of immune phage antiserum and the homologous phage will result in a continuous decrease in plaque forming units (PFU = infectious phages) with a very high Kn value. The same immune phage serum mixed with a different but closely related phage will result in less efficient serum neutralization with a smaller Kn value. The same immune phage serum mentioned above mixed with an unrelated phage will not result in serum neutralization, therefore the Kn will be zero.

Burnet, Koegh, and Lush (7) presented the mathematical measurements for the rate of phage inactivation, as represented by the following equation:

Eq. 1

$$-\frac{dP}{dt} = \frac{KnP}{D}$$

Integration of equation 1 yields:

Eq. 2

$$\log \frac{P}{Po} = -\frac{Knt}{2.3D}$$

Equation 2 expressed the observations that the logarithm of the fraction of surviving phages (P/Po) decreases with time, and the slope of this survival curve (Kn) is inversely proportional to the reciprocal of the dilution factor of the antiserum D, used in the reaction mixture. To solve for Kn the equation is:

Eq. 3 $$Kn = \frac{2.3D}{t} \log \frac{Po}{P}$$

Kn = neutralization rate constant ($min^{-1}$ 1 L per min)

D = reciprocal of dilution factor of serum; if antiserum is 1:100 dilution; the D = 100

t = time in minutes

Po = phage titer at time 0 (PFU/ml)

P = phage titer at time t (PFU/ml)

The constant Kn determined under standard conditions is called the titer of the antiserum used. If a serum neutralization test between anti-T4 and T4 showed Kn = 500 $min^{-1}$, then the titer of the antiserum is 500 per min, which indirectly describes the activity of antibodies present by quantitating the number of particles being neutralized per minute.

The conventional assay method for the detection of antibody activity was illustrated by the bacteriophage neutralization test described by Adams (1), employing the soft agar overlay techniques in plating replicate samples of phage-antiserum mixtures. The number of plaque-forming units was observed to decrease as a function of time. In the following experiment you will follow the kinetics of serum neutralization by diluting samples of phage-antiserum mix at different time intervals after mixing. These diluted samples will be plated on sensitive E. coli indicator in order to assay the number of particles still infectious (not neutralized). The number of plaques counted will equal the number of phages not yet neutralized for two reasons: (1) dilution of mixture at specified time intervals stops the serum neutralization occurring in that sample immediately and (2) the reaction of phage with neutralizing antibody is an irreversible reaction, i.e. the neutralized particles aren't suddenly going to become active while you're plating the samples. Calculations derived from relationships established by Equation 3 (7) allowed evaluation of the effectiveness of serum samples for phage inactivation, as denoted by the value of Kn. This assay reflected the presence of those antibodies primarily responsible for neutralization of several phage antigens, in particular, those of the tail fibers.

# KINETICS OF SERUM NEUTRALIZATION

Materials: (PER GROUP OF 4)

phage stocks: 1.0 ml of each of the following at a titer of ≈ $10^7$ PFU/ml T1, T2, T4, T7

| | |
|---|---|
| tubes of 1.8 ml of antiserum dilution in 3" tube (dilution will be given) | 4 ea |
| 24 hr *E. coli* in 7" tube | 18 ml |
| 9.9 ml dilution blank (1:4 PA:saline) | 28 ea |
| 4.5 ml dilution blank (1:4 PA:saline) | 43 ea |
| 1.0 ml pipettes | 60 |
| 0.1 ml pipettes | 26 |
| pasteur pipettes | 40 |
| plates (sterile) | 92 |
| 7.5 ml of tryptose-phosphate agar in 7" tube | 92 |

Method: Assay using pour plate method instead of overlay

Student 1 - Antiserum vs T1

Student 2 - Antiserum vs T2

Student 3 - Antiserum vs T4

Student 4 - Antiserum vs T7

All students are responsible for recording and graphing data of all 4 phage-antiserum mixes.

1. Before making any of the phage-antiserum mixes assay each of the phage stocks. All assay plates will be done by pour-plate method using 7.5 ml of tryptose-phosphate soft agar tubes, as follows:
   a. Make dilutions as usual. You will need to plate $10^{-4}$ and $10^{-5}$

dilutions for T4 and T2, using two 9.9 ml blanks and one 4.5 ml blank. Plate $10^{-}$ and $10^{-}$ for T1 and T7. Do this by using only two 9.9 ml blanks and two 4.5 ml blanks, you will make duplicate plates of each dilution to be plated.

b. Seed each tryptose-phosphate soft agar tube; kept at 45 C with 8 drops of 24 hr indicator using a pasteur pipette.

c. To each of two soft agar tubes which are already seeded with _E_. _coli_ using a 1.0 ml pipette, put 0.5 ml of proper tube dilution.

d. Immediately mix these soft agar tubes thoroughly and as quickly as possible pour the _entire_ contents of tube into a sterile empty petri dish. Swirl the plate to distribute agar evenly. Allow plates to solidify for 20 minutes before incubation at 37 C.

e. NOTE: You are plating 0.5 ml! Make the appropriate changes in your calculations.

2. Each series of titrations, e.g. antiserum B vs T4 will be performed by the following procedure except for substitution of a different antiserum and phage.

3. At time 0 add 0.2 ml of the proper undiluted phage stock to a tube containing 1.8 ml of designated antiserum (this is a $10^{-1}$ dilution of undiluted phage stock) that has been incubated at 37 C. Mix well.

_FOR T7 AND T1_

4. Immediately transfer 0.1 ml of this phage-antiserum mixture to a chilled 9.9 ml dilution blank and keep on ice. This is a $10^{-2}$

dilution of phage antisera mix at zero time. Make a $10^{-4}$ and $10^{-5}$ dilution using chilled 9.9 and 4.5 ml blanks. Return the mix to 37 C water bath immediately and maintain this temperature during 30 min sampling period.

Plate the last two tube dilutions, $10^{-4}$ and $10^{-5}$ using pour-plate method. Remember to add 8 drops of indicator before adding 0.5 ml of proper tube dilution.

5. Repeat step 4 at each sample time, 3', 10' and 30' for each phage-antiserum mixture. Except at this time make only $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions and plate in duplicate the $10^{-3}$ and $10^{-4}$ tubes.

FOR T2

4. Transfer 0.1 ml of the phage-antiserum mixture and make a $10^{-2}$ dilution using a 9.9 ml dilution blank. Make a $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilution using chilled 9.9 and two 4.5 ml blanks. Return the mix to the 37 C water bath immediately and maintain the temperature during the sampling period. Plate the last three dilutions $10^{-4}$, $10^{-5}$ and $10^{-6}$ using pour plate method.
5. Repeat step 4 at each sample time except at 3 and 10 min make $10^{-2}$, $10^{-4}$ and $10^{-5}$ dilution and plate the last two and at 30 min make $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions and plate all three.

FOR T4

4. Do as for T2. Plate $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions at 0 minutes.
5. Repeat step 4 but at 3 minutes make $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ dilutions, plate the last three. At 10 minutes, make $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions and plate all three. At 30 minutes make

$10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions and plate all three.

6. Incubate all plates at 37 C for 15-18 hrs. Calculate the titer of active phages for each time; PFU/ml of mix.

Data section will include all plates counts, titers of active phages at each time, Kn for each time in the following data tables; there will be 1 table for each mix.

Table 1: Antiserum A vs T1

| Time | $5 \times 10^{-4}$ Tube Dilutes | $5 \times 10^{-5}$ Recorded | $5 \times 10^{-6}$ on Plate | Titer of Active Phages PFU/ml | Titer of Antiserum Kn Value ($min^{-1}$) | P/Po |
|---|---|---|---|---|---|---|
| 0 | | | | | | |
| . | | | | | | |
| . | | | | = Po | | |
| . | | | | | | |
| . | | | | = P | | |
| . | | | | | | |
| . | | | | | | |
| 30 | | | | | | |

Table 2: Antiserum A vs T4

Table 3: Antiserum A vs T2

Table 4: Antiserum B vs T4

The data sections will also include 1 graph. In graph, plot the number of survivors (P/Po) on the verticle axis of 3-cycle semilog paper vs time on the horizontal axis; for each $\phi$ type, 4 lines on same paper. Label each with phage type.

Compare and describe the differences in shape of the four neutralization curves. Conclusions: (1) What is the homologous phage of antiserum B, i.e. against what phage is this antiserum specifically directed? (2) Using the Kn values what conclusions can be drawn about the degree of serological relatedness of each phage to the other three phages. Each member of the group must turn in his/her own conclusions and graphs of all four phage.

Questions

1. In your experiment there was no serum control as there should have been. Describe exactly what the control would consist of, what it would show, and how that data would be used. Be sure to include a brief explanation of the kind of serum which would be used. (Hint: reread paragraph 5).
2. Group these four T phages (T1, T2, T4, T7) into two distinct groups based on morphology of their plaque. Include a description of each plaque type [shape, size (measure in mm), degree of turbidity within plaque, and absence or presence of a halo]. How does this grouping of T1, T2, T4, T7 compare with your grouping of them based on serological relatedness?

## PHAGE-RESISTANT MUTANTS

A mutation may be defined as a permanent and heritable change of a cell property which arises spontaneously through alterations in the cell genetic material (1). Such modifications provide for the development of new classes of variants, some of which represent stable forms, others which are less stable and revert. In a bacterial population, spontaneous mutations occur during normal growth and it is possible to find cells that have acquired resistance to bacteriophage infection (4). The presence of these variants has been investigated and confirmed (11). Conventional designations for phage-resistant bacterial mutants were established by Burnet and McKie (5). A diagonal is placed between the symbol representing the sensitive parent and the phage type to which it is resistant. For example, B/2 indicates a strain of *Escherichia coli* β resistant to bacteriophage T2. A mutation which imparts simultaneous resistance for several phages, e.g., T4 and T7, is designated as B/4,7. The mutational profile (1) of *Escherichia coli* β for the T-phages has been described (6). The natural frequency of spontaneous mutation in a normal, growing culture, i.e., the mutational rate per bacterium per generation, was observable in a range from $10^{-7}$ to $10^{-9}$.

To detect these mutants, a simple method employed the plating of the bacterial culture in excess of bacteriophages; all susceptible cells were lysed except for colonies which contain phage-resistant cells (11). These were restreaked and reisolated for purity and retested for resistance to the phage to which the parent strain was sensitive. The spontaneous rate has been determined to represent a very low frequency for some phages, e.g., T2, where occurrence of such mutations is rare. Various methods may be employed to increase the recovery of mutants in a culture. For example, large volumes of a phage-infected culture may be concentrated and plated for resistant colonies (1). Alternatively, experimental induction methods have utilized different chemical and physical agents. One mutagenic agent, nitrosoguanidine, commonly used in genetic studies was first reported for its mutagenic effect in _E. coli_ by Mandell and Greenberg (12). Levels of the agent were incorporated into the agar layer before spreading the phage-infected culture. Broth cultures may be incubated in the presence of nitrosoguanidine for various time intervals prior to phage infection and plating (2,13). Other methods employing radiation treatments of UV or X-rays have also been investigated to various extents (3,7,10,15).

A recent investigation has reported the effective use of trypsin to enhance the isolation of phage-resistant, lytic-sensitive mutants, especially of the N-phage series (9). The procedure involved the addition of trypsin to the growth medium, a level that did not appear to affect growth rate or phage adsorption. The presence of trypsin has also been observed to increase phage yields several magnitudes higher when compared to cultures containing no trypsin. It may be possible to apply the use of this agent to other phage-host systems to determine whether similar results can be obtained.

Phage-resistant mutants have proved to be a useful technical tool as effective indicator strains for the detection of one virus type in a mixture of two or more phage strains (1). They aid in screening host range phage mutants as well, i.e., those phages in a culture capable of infecting the resistant mutants themselves. As bacterial mutants have provided utility in viral genetic research, observations have also been extended and applied in similar fashion to bacteriophage mutants. The principles underlying viral mutations and genetics have proven to be varied and complex. Snustad and Dean (14) have designed and presented selected experiments to provide an introductory basis for student understanding and exploration of important aspects in virus mutation.

## PHAGE-RESISTANT MUTANTS

**Cultures**: PER PAIR OF STUDENTS

| | |
|---|---|
| *E. coli* β (2.5 hours) | 6.0 ml |
| Phage stocks ($>10^{10}$ PFU/ml) T1, T2, T4, T7 | 1.5 ml each phage |

**Materials**:

| | |
|---|---|
| base agar plates -- 5 per each phage tested | 20 |
| sterile dilution tube | 4 |
| glass L-rod, one for each phage type | 4 |
| ethanol and beaker | 4 |
| sterile 10 ml pipette | 2 |
| sterile 1.0 ml pipette | 6 |

| To be supplied during week of testing and isolation: | PER STUDENT |
|---|---|
| agar slants | 5 |
| base agar plates | 10 |
| soft agar overlays (2.5 ml) | 10 |
| phage stocks for spot tests | 2 sets of bottles, containing 15 mls of the four phage types |

Pasteur pipettes and bulbs for spotting plates

Optional materials: PER CLASS

glass slides

Gram stains

inoculating loops

base agar plates for restreaking

microscope for slides

stereoscope for colonial morphology

This experiment is an attempt to isolate spontaneous phage resistant mutants from a phage-infected culture and to investigate certain characteristics of mutant resistance. It will be divided into three sections and conducted over a two laboratory session period.

### Procedures:

A. Isolation and Identification of Phage-Resistant Mutants:

1. This experiment will be done in pairs, in which one student will use T1 and T4 phages, and the second student, T2 and T7 phages. All observations and data will be combined and compared.
2. Follow the given procedures for each phage type tested:
    - (a) Mix 1 ml of a 2.5 hour *E. coli* β culture with 1 ml of the designated phage (virus/cell ratio should be approximately 50-100:1).
    - (b) Shake the mixture and incubate at 37 C for 10 min. Remove the culture and plate 0.2 ml to each of 5 base agar plates by the spreading technique. Allow to dry.
    - (c) Incubate the plates for 18-24 hours. Examine for the numbers and colonial morphology of the resistant colonies. Record and describe.
    - (d) Choose 5 well-isolated resistant colonies from each set of 5 plates. It may be best to prepare a gram stain on each

colony tested to check for purity. If necessary, restreak on another agar plate until colonies are pure, isolated, and free from phage. Recheck again with gram stain. Use only a very small inoculum for the slide preparation.

(e) Streak a minimal portion of the pure colony to an agar slant. Incubate at 37 C for 18-24 hours. Then store in the refrigerator. This will serve as the stock culture for later experiments. Inoculate the remaining portion of the colony into a tube of soft agar. Mix gently and overlay. Allow the agar to set for 15 min., making sure the overlay is uniform and level. Mark the plate into 4 quadrants, labeled T1, T2, T4, and T7. Taking a sterile Pasteur pipette, carefully spot with the appropriate phage, using the smallest drop possible to avoid overlapping or running into the other quadrants. Use a separate pipette for each phage type.

(f) Allow the plates to adsorb for 20-30 min. Incubate at 37 C for 18-24 hours, leaving the plates right side up. DO NOT INVERT.

(g) Read the results and record sensitivity to resistance to each phage type.

(h) Identify the mutant culture by designation, e.g., culture resistant to phage T2 will be written as B/2, or to T4 and T7, as B/4,7, and so forth.

3. Repeat the spot check for the slant cultures and compare to the first set of results.

4. Submit the slant cultures labeled appropriately with name, date isolated, and the designation of the mutant. Retain one culture for use in subsequent experiments.

## PHAGE-RESISTANT MUTANTS

### Host Resistance

Two critical steps in productive bacteriophage infection are the adsorption of the virus to the susceptible host, followed by subsequent intracellular multiplication within the host cell. Adsorption occurs as a result of complementarity of surface structures of the virus and host, such that the appropriate reaction can proceed (4,2). The physiological basis of host resistance may be attributable to alterations in the cell surface makeup, especially modifications of receptor sites at or near the point of attachment for the specific phages. The result is primarily loss of adsorptive capacity of phages to host cells.

Changes may be due to direct mutation in the host cell genetic material or due to effects by cultural conditions, e.g., temperature, pH, enzymatic action, or ionic factors, which contribute to altering the cell receptor specificity.

## PHAGE-RESISTANT MUTANTS

Cultures: PER STUDENT

| | |
|---|---|
| *E. coli* β indicator (24 hours) | 8 ml |
| *E. coli* β culture (2.5 hours) | 4 ml |
| Resistant mutant culture (2.5 hours) to be prepared by student | 4 ml |
| Phage stocks (diluted to $10^7$ PFU/ml) | 1.5 ml of designated phage type |

Materials: PER STUDENT

For day prior to lab:

| | |
|---|---|
| sterile 125 ml Erlenmeyer flask | 1 ea |
| sterile broth medium | 10 ml |
| inoculating loop | 1 ea |

For day of the lab (2.5 hrs. before):

| | |
|---|---|
| sterile 125 ml Erlenmeyer flask | 1 ea |
| sterile broth medium | 20 ml |
| sterile 1.0 ml pipette | 1 ea |
| soft agar overlay | 30 ea |
| sterile petri plates | 30 ea |
| chloroform | 2 ml |
| 0.04 *M* KCN | 1 ml |
| sterile dilution tubes | 30 ea |
| sterile chilled diluent | 175 ml |

| | |
|---|---|
| sterile 10 ml pipette | 5 ea |
| sterile 1.0 ml pipette | 40 ea |
| ice bath | |

Procedures:

B. Determination of Host Resistance:

The day before the lab session, inoculate from your mutant culture slant into 10 mls of broth contained in a sterile flask (125 Erlenmeyer). Incubate on shaker at 37 C overnight.

On the day of the lab 2.5 hrs before, prepare a young culture by diluting the overnight culture 1:200 into 20 mls of warm broth (0.1 ml into 20 ml). Shake for 2.5 hrs at 37 C for use in lab.

The *E. coli* β indicator and experimental cultures will be already prepared and ready for use.

1. Prepare each of the following reaction tubes:
   - (a) 1.6 ml *E. coli* β + 0.2 ml diluent broth,
   - (b) 1.6 ml *E. coli* β + 0.2 ml KCN (0.04 *M*),
   - (c) 1.6 ml mutant culture + 0.2 ml diluent broth,
   - (d) 1.6 ml mutant culture + 0.2 ml KCN (0.04 *M*),
   - (e) 1.8 ml diluent broth

   Allow to react for 5 minutes.
2. To each tube add 0.2 ml of phage (corresponding to the mutant strain used).
3. Incubate at 37 C for 30 minutes.
4. Add 0.5 ml of chloroform to each tube. Shake vigorously. Allow layers to separate in ice bath.
5. Assay the aqueous layers using the 7.5 ml soft agar overlay for the following dilutions:

(a) $10^{-3}$, $10^{-4}$, $10^{-5}$

(b) $10^{-1}$, $10^{-2}$, $10^{-3}$

(c) $10^{-3}$, $10^{-4}$, $10^{-5}$

(d) $10^{-3}$, $10^{-4}$, $10^{-5}$

(e) $10^{-3}$, $10^{-4}$, $10^{-5}$

6. Incubate all plates 18-24 hrs. Count plaques and determine the number of PFU per ml for each set.

   Make comparisons and observe the significant results from each experimental tube.

   Draw conclusions as to the nature of host resistance to phage.

## PHAGE-RESISTANT MUTANTS

### Mixed Indicators:

Phage-resistant mutants have served as useful selective assay tools in detection of one virus type in a mixture of two or more viruses. As observed, the mutant is usually completely resistant to one specific phage, but susceptible to a second virus. One technique involved is the use of mixed indicators, a technique developed by Delbruck (1) which permitted the assay of two phage types in a single plating of a phage suspension. Equal parts of the sensitive bacterial culture and the resistant mutant culture are inoculated and serve as a composite indicator organism culture. The noteworthy feature in the assay is that all plaques appear turbid as a result of lysis of the sensitive strain and growth of the resistant strain (2). This then serves as an index for identification of the virus type.

# PHAGE-RESISTANT MUTANTS

Cultures: PER STUDENT

| | |
|---|---|
| *E. coli* β indicator (24 hrs) | 5 ml |
| Resistant mutant isolated (24 hrs) | 5 ml |
| Phage stocks (diluted to $10^7$ PFU/ml) | 1 ml of phage type according to mutant<br>1 ml of another phage |

Materials: PER STUDENT

Day prior to lab:

| | |
|---|---|
| sterile 125 ml Erlenmeyer flask | 1 ea |
| sterile broth medium | 10 ml |
| inoculating loop | 1 ea |

Day of the lab:

| | |
|---|---|
| basal agar plates | 36 ea |
| soft agar overlay (2.5 ml) | 36 ea |
| sterile chilled diluent | 100 mls |
| sterile 10 ml pipette | 3 ea |
| sterile 1.0 ml pipette | 20 ea |
| ice bath | |

Procedures:

C. Mixed Indicator Assay:

The day prior to the laboratory session, inoculate from mutant stock culture into a sterile 125 ml Erlenmeyer flask containing

10 mls of sterile broth. Incubate on shaker at 37 C overnight for use as an indicator culture.

The *E. coli* β indicator (24 hrs) will be prepared and supplied for use in the lab.

1. Select the phage stock corresponding to your resistant mutant and a second phage type to which both cultures will be susceptible.

   Dilute each stock appropriately to $10^{-6}$ dilution. Assay from $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions for the experiment as indicated below, using 2 replicate plates per dilution:

   (a) *E. coli* β indicator (0.1 ml) + 0.1 ml phage dilution;

   (b) Resistant mutant (0.1 ml) + 0.1 ml phage dilution;

   (c) *E. coli* β indicator (0.05 ml) + 0.05 ml resistant mutant indicator + 0.1 ml phage dilution.

2. Repeat the above for the second phage type.

3. Allow the soft agar to solidify. Incubate all plates 18-24 hrs.

4. Count plaques and determine the phage titer for each.

5. Compare and comment on differences. Be sure to note in particular the appearance of plaque morphology in each assay.

# LYSOGENY

A virulent phage such as the T phages adsorbs, infects, and initiates lysis of the susceptible host cell releasing many phage progeny (lytic cycle). Another relationship between a phage and a host cell (and probably more prevalent) is lysogeny in which a temperate phage adsorbs to and infects a susceptible cell. After infection by a temperate phage there are two possible cell responses depending on the physiological status of the infected cell: (1) lytic response, the entering phage enters the vegetative state, multiplies and forms mature progeny phages which are eventually released by cell lysis; (2) lysogenic response, the entering phage genome (nucleic acid molecule) is repressed and enters the prophage state which permits the phage genome to exist unharmed in the cell but no infectious progeny particles are formed so the infected cell continues to metabolize and divide as a lysogenic bacterium. In lysogeny the infecting phage is maintained as a non-infectious unit of phage nucleic acid devoid of any structural proteins. The prophage can exist in two states usually dependent on the infecting phage type : (1) chromosomal state, the prophage has totally integrated into the host chromosome and is replicated in unison with the host chromosome, thus all the progeny cells of a lysogenic cell of this type carry the genetic potential to produce phage particles, (2) cytoplasmic state, the prophage exists as a DNA molecule independent of the host chromosome and replicates at a rate slower than the cell divides. Some of the daughter cells of this type of lysogen will loose the ability to produce phage particles.

Lambda (λ) is an example of a temperate phage which can infect *E. coli* K12 with either of the two above responses. The lysogen is designated by following the name of the bacterium with the temperate phage establishing lysogeny in parentheses, e.g., *E. coli* K12 (λ) indicates that *E. coli* K12 has been lysogenized by λ. The prophage of λ exists in the lysogen in the integrated chromosomal form. For the λ system, there is evidence to suggest that major determinant of the fate of λ when it infects a susceptible cell (K12) is the physiological state of the cell. A cell which is actively metabolizing and healthy will give way to a lytic response upon initial infection by λ. Alternatively, a cell which is physiologically unhealthy will give way to a lysogenic response, a "sick" cell is one that will probably not yield many progeny phages and it is to the infecting phage's advantage to wait around for better cell conditions in order to get the maximum burst size. After all, no self-respecting phage wants to replicate in a cell that's over the hill.

A lysogenic cell shows almost no outward appearance of the "symbiotic relationship between the cell and the phage except for the phenomenon of *lysogenic immunity*. Lysogenic bacteria are immune to superinfection and lysis by the same phage type as the prophage it carries. If this were not true then the phenomenon of lysogeny could never have been observed. Immunity is distinct from phage resistance because lysogenic cells are still able to adsorb the homologous temperate phage type (compare this to the nature of T-phage resistance in *E. coli* β). Immunity is very specific since one strain of $\lambda_i^{435}$ phage may not be able to superinfect a lysogen of E. coli K12 ($\lambda_i^{435}$) but the wild type $\lambda^+$ would be able to superinfect E. coli K12 ($\lambda_i^{435}$). The characteristic for immunity is coded for by a small region of the phage genome which is one of the few regions of the prophage which is functionally expressed in the repressed prophage (another region which is expressed as the area coding for the repressor molecule).

During cell multiplication some types of lysogenic bacteria will enter the lytic cycle, spontaneously releasing phage. In others, this genetic prophage is more stable and the presence of free phage within the cultures is rare, i.e. the spontaneous background level is low. There are several physical and chemical agents (ultraviolet, X-ray, nitrogen mustard, etc.) that will induce the lysogenic cell to enter the lytic cycle. A susceptible indicator organism must be used in assaying for the released phage particles, this must be a cell type that the temperate phage can lytically infect. In other words it cannot be a cell strain which is immune to infection by this phage type nor can it be a cell strain which is resistant to infection by this phage type. For the λ experiments here E. coli K12 will be the indicator organism. When the λ prophage is induced to enter the lytic cycle, it has become excised from the chromosome and it enters the vegetative state eventually causing the lysis of the cell to release new λ progeny. The event of λ excision from the chromosome can be thought of as a consequence of derepression of the repressed prophage.

Materials: PER GROUP OF 3

| | |
|---|---|
| 9.9 ml blanks (1:4 saline to PA dilution blanks) | 9 ea |
| 4.5 ml blanks (1:4 saline to PA dilution blanks) | 13 ea |
| soft agar tubes -- 7.5 ml of Tryptose-phosphate soft agar | 36 ea |
| sterile plastic petri dishes | 36 ea |
| sterile glass petri dish | 1 ea |
| sterile screw cap tube | 1 ea |
| sterile 5" test tube | |
| 1.0 ml pipettes (used wherever you have to pipette .25 or 15 ml samples) | 20 ea |
| 5.0 ml pipettes | 1 ea |
| 10.0 ml pipettes | 2 ea |
| 0.1 ml pipettes | 9 ea |
| pasteur pipettes | 12 ea |
| ml of K12 (λ) 2.5 hr culture suspended in M-9 medium in 7" tube | 13 ml |
| ml of K12 indicator culture in 7" tube | 20 ml |
| centrifuge | |
| aluminum foil | |
| UV light | |

Method: This experiment will be done in groups of 3.

A. Student #1 titer of lysogenic cells before UV treatment.

1. Plate nonirradiated K12 (λ) culture at various dilutions using pour plate method. Put 0.5 ml of tube dilution into soft agar tube and do not use indicator, since you want a colony count.

Make duplicate plates of the tube dilutions $10^{-5}$, $10^{-6}$, $10^{-7}$. The titer calculated from these plates will be equal to the total number of viable lysogenic cells in this 2.5 hr K12 (λ) culture before UV treatment (No).

B. Student #2 control-spontaneous background level of free λ particles.

2. Plate nonirradiated K12 (λ) culture at various dilutions using pour plate method, put 0.5 ml of dilution into soft agar tube previously seeded with 6 drops of indicator K12. Make duplicate plates of undiluted, $10^{-1}$, and $10^{-2}$ samples. The titer of PFU's calculated from these plates will equal the number of free infectious λ found in the medium surrounding the lysogenic cells, (PSB). Assume that this value is constant.

   Explain why these free infectious λ particles exist in the immediate surroundings of these cells (K12 [λ]) without causing clearing of the cell suspension as would the T phages?

C. Students #1, 2, 3 UV induction of K12 (λ) cells.

3. Student #3 pipette 8 ml of nonirradiated K12 (λ) culture into a sterile petri dish. [The K12 (λ) is suspended in synthetic medium M-9 which is colorless.Why is the medium used for irradiating the cells?] Irradiate the culture for 2 seconds with the glass lid off. Why is it necessary to remove the glass lid? Immediately afterwards pipette into a foil covered screw cap tube with a 10 ml pipette this irradiated culture. This tube must be covered at all times with foil to block out any light. This will prevent photoreactivation repair of thymidine dimers in

the DNA caused by the UV.

4. Student #3 will assay the irradiated culture immediately after it is put into the foiled tube. Using the pour plate method pipette 0.5 ml of tube dilution into each soft agar tube which has been previously seeded with 6 drops of indicator K12. Make duplicate plates of $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ dilutions. The titer calculated from this infective assay of the UV culture with no incubation (ICo) is equal to the number of UV induced cells (Nuvi) plus the spontaneous background level of λ(PSB). To calculate the number of UV-induced cells:

Equation 1

$$\text{Nuvi} = \text{ICo} - \text{PSB}$$

To calculate the fraction of K12 (λ) cells which were UV induced:

Equation 2

$$\text{fuv} = \frac{\text{Nuvi}}{\text{No}}$$

5. Student #2 will assay the irradiated culture immediately after it is put into the foil covered tube in the following way. Using pour plate method pipette 0.5 ml of proper tube dilution, into each soft agar tube with no indicator. Make duplicate plates of $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ dilutions. The titer calculated from those colony counts represents the number of viable noninduced lysogenic cells which survive the UV treatment (Nni) to calculate the total number of cells which survive (Nts).

Equation 3 $$\text{Nts} = \text{Nni} + \text{Nuvi}$$

To calculate the percent of the total K12 ($\lambda$) cells that survive the UV treatment:

Equation 4 $$\text{survival rate} = \frac{Nts}{No} \times 100$$

6. As soon as possible student #1 should put the foil covered tube in the 37 C incubator. Incubate for 90 min, to allow time for all the induced cells to replicate and release progeny $\lambda$.
7. After 90 min incubation period student #2 needs to centrifuge the lysate (remove the foil in order to get tube into centrifuge). Being careful not to shake the separated layers transfer about 3 ml of the supernatant only to a sterile 5" tube to be used by students 3 & 1 below. The pellet contains all debris, viable noninduced K12 ($\lambda$), and dead noninduced K12 ($\lambda$) cells. The supernatant contain infectious $\lambda$ particles.
8. Student #1 will assay the above supernatant for $\lambda$ particles. Make duplicate plates of the tube dilutions $10^{-6}$, $10^{-7}$, $10^{-8}$ by pipetting 0.5 ml of the tube dilution into a soft agar tube previously seeded with 6 drops of indicator K12. The titer of PFU calculated from this infective center assay of the UV culture after 90 min incubation (IC90) equals the number of $\lambda$ released by UV induced cells and the spontaneous background level of free $\lambda$. To calculate the average number of $\lambda$ released per UV induced cell:

Equation 5 $$\text{burst size} = \frac{IC90 - P56}{Nuvi} = \frac{\text{number of } \lambda \text{ released from UV induced cell}}{\text{number of UV induced cell}}$$

Assuming that this is a constant value for any lysogenic K12 ($\lambda$) cell calculate how many cells are responsible for the presence of the spontaneous background level of free $\lambda$ (P56).

Equation 6

$$\frac{\text{P56}}{\text{burst size}} = \begin{array}{l}\text{\# of cells which were spontaneously} \\ \text{induced to enter the lytic cycle}\end{array}$$

Why don't the progeny $\lambda$ particles newly released reinfect other nearby cells during the 90 min incubation period causing the release of more and more $\lambda$? In other words, why can we assume that all the $\lambda$ progeny are the result of only one round of lytic cycle from the UV induced cells and thus be able to calculate the burst size with reasonable accuracy?

D. Student #3 Demonstration of Lysogenic Immunity.

9. To each of two soft agar tubes pipette .25 ml of the original nonirradiated K12 ($\lambda$) (leftover from step 3) and .25 ml of the supernatant obtained in step 7. Mix and pour into separate plates. Count the number of plaques, each student is to record and explain the presence or absence of plaques on these two plates.

The following data table will be compiled by each student from data of all 3 group members. (Table on next page)

Data Table: Lysogeny

| Value to be Determined | Plate Counts *Tube Dilutions to be Filled In | Titer |
|---|---|---|
| Total # of viable K12 (λ) cells before UV treatment | * | No = |
| Spontaneous background level of free λ | * | PSB = |
| Infective center assay of UV culture with no incubation | * | ICo = |
| Titer of UV induced cell | Eq. 1 | Nuvi = |
| Fraction of K12 (λ) cells which were induced by UV | Eq. 2 | fuv = |
| Titer of noninduced viable K12 (λ) cells surviving UV | * | Nni = |
| Total number of cells surviving the UV treatment | Eq. 3 | Nts = |
| Survival rate | Eq. 4 | |
| Infective center assay of UV culture after 90 min at 37 C | * | IC90 = |
| Burst size for λ | Eq. 5 | |
| Number of K12 (λ) cells which enter lytic cycle spontaneously | Eq. 6 | |

Questions:

1. See step #2.
2. (a) See step #3.
   (b) See step #3.

3. Calculate the number of cells which were killed by UV treatment.
4. See step #8.
5. Explain the difference in plaque counts between plates made in step 9 and those made in step 8.
6. Postulate how UV irradiation increases the number of cells which enter into the lytic infective cycle.
7. Describe the morphology of the λ plaques on K12 indicator (size, shape, degree of turbidity, presence of absence of halo, etc.). In what characteristic does it significantly differ from all of the T plaques and why?

# T4 MORPHOGENESIS: IN VITRO COMPLEMENTATION BETWEEN DIFFERENT AMBER MUTANTS OF T4

Conditional lethal mutants fall into four groups: temperature-sensitive (ts), amber (am), ochre, and azure. The ts mutants are characterized by their ability to form plaques at 25 C (permissive) but not at 42 C (restrictive or nonpermissive). The mutated gene in a ts mutant codes for a "mutant" protein that functions normally at 25 C, but becomes inactivated at 42 C thus preventing phage propagation at the elevated temperature if this mutation has occurred in an essential gene. This has been found to be caused by a single base change in the gene causing the appearance of different codon in the mRNA for that gene. This change in codon results in the substitution of a wrong amino acid when mRNA is being translated into the protein. Since the ts mutation is present but not expressed at 25 C, this permissive temperature must be used to isolate and propagate stocks of ts mutants.

The amber mutants were initially characterized by their ability to grow lytically on certain strains of *E. coli* but not on others. The permissive strains of *E. coli*, those that support propagation of am mutants, are designated phenotypically $Su^+$. The nonpermissive strains of *E. coli* do not support the propagation of am mutants and are phenotypically designated $Su^-$. Amber mutants result from a single base substitution in a gene that changes a mRNA codon for an amino acid to UAG, a termination codon. Since this UAG codon appears somewhere in the middle of the gene's mRNA, when it is translated in a nonpermissive cell ($Su^-$) instead of plugging in the proper amino acid as the normal codon would have, the UAG codon stops the elongation of that polypeptide chain. This "nonsense" mutation, when expressed, results in the formation of an incomplete polypeptide which is nonfunctional because the rest of the normal codons in that mRNA following the UAG amber codon are not translated. The fact that phage am mutants can grow in certain permissive cells means that these amber nonsense mutations are suppressible. The permissive cells are able to support propagation of am phage mutants because they possess a species of tRNA not found in the nonpermissive cells. This different species of tRNA mis-reads the UAG amber codon as a codon for an amino acid; therefore the polypeptide chain synthesis is continued without early termination resulting in the formation of the functional protein and allowing the am phage to replicate in the $Su^+$ cells just like a wild type phage. A suppressor mutation by definition is one that occurs in a location other than the original mutation, but causes the phenotypic loss of the original mutation.

The ochre and azure mutations are suppressible nonsense mutation just like the am mutation; but, whereas the nonsense codon for amber is UAG, the nonsense codon for ochre is UAA and for azure mutants it is UGA. In λ the amber mutation (UAG) is called sus, e.g. λ sus $C_I$.

As in the ts mutants these am, ochre, and azure conditional lethal mutants can only be isolated and propagated in permissive conditions ($Su^+$ cells) so that the lethal nonsense mutation is not expressed even though it is present. The lethal nature of the mutation is seen by infection of a nonpermissive host where no infectious phage progeny are made, i.e. the lethal nature is conditional, only seen under certain conditions.

The use of conditional lethal mutants of T4 has not only helped to map the position of the different genes, but it has also helped to uncover the exact function that those gene products are responsible for during the production of phage particles. Conditional lethal mutations can occur in the early genes which are concerned with the inhibition of host macromolecule synthesis, the synthesis and regulation of phage DNA synthesis and lysozyme synthesis, but for the purposes of the following discussion of T4 morphogenesis these will be ignored.

Viral morphogenesis may be defined as the process by which the completed infectious unit is assembled from its previously synthesized component parts. The particular mutations you will use in the lab are all amber mutations which effect morphogenesis (maturation) of the T4 phage at various stages. The late genes are primarily the maturation genes, and at least 40 different genes are required for T4 assembly. Included within this class are genes coding for head morphology (e.g., gene 23), tail, sheath, and tail fiber protein components. When various am mutants are grown in nonpermissive cells no mature particles are assembled, but the cytoplasm of these cells do contain a large accumulation of recognizable parts of the phage, except for the one effected by the amber mutation. Therefore, Su$^-$ extracts made in the way described in experiment IIA will contain heads with no tails if the am mutation is in a tail gene, or heads with tails but no tail fibers (e.g. T4 am N52, B252, and X4E), or complete tails but no heads (e.g. T4amB17).

W. B. Wood (1966) has elucidated the functions of some of these morphogenic genes, specifically those involved with tail fiber formation by the study of complementation between morphogenic amber mutants in vitro. When Su$^-$ extracts containing particles without tail fibers are mixed in vitro with Su$^-$ extracts containing normal phage tail fibers, the two components combine (the head-tail assembly combines with the tail fiber) to form infectious pahge. Thus, the tail fiber components present in the extracts of nonpermissive cells infected with various tail-fiberless amber mutants can be analyzed by their ability to complement each other in vitro. Su$^-$ extracts of am mutants with different maturation gene defective will complement each other when mixed in vitro, thus resulting in the in vitro morphogenesis of active phage. The detection of infectious particles from such a mix indicates that complementation has occurred. When Su$^-$ extracts of amber mutation with defects in the same maturation gene will not complement each other and no infectious phage can be detected, complementation did not occur.

Conditional lethal mutants are just one of a large number of different groups of mutations that have been extensively used, for the analysis of phage genetics. Ever since their discovery in λ (Campbell 1961) and T-even phages (Epstein 1963; Edgar 1964) they have become increasingly more important for the fine structure analysis of these phage genomes for the following reasons: (1) Conditional lethal mutations are not restricted to any one set of genes, and theoretically almost every gene in the genome can be effected, thus allowing complete mapping. (2) Since these mutations in any gene can be isolated and propagated under permissive conditions, mutation effecting essential genes can be analyzed by following the course of infection under non-permissive conditions where the lethal genetic defect shows up. This allows the analysis of essential biosynthetic or morphogenic steps which can not be done with other types of nonconditional lethal mutations. Plaque morphology mutants (r) and host-range mutants (h) are not mutants in essential functions since the phage can still propagate when the mutation is expressed (by definition an essential gene function is one that when mutation in that gene is expressed there is no productive replication). Conditional lethal mutants can propagate under permissive conditions but just like other types of mutations in essential gene functions when the conditional lethal mutation is expressed (non-permissive conditions) the phage can not propagate. (3) Complementation tests can be carried out between two conditional lethal mutants to determine if the conditional lethal mutations occur in the same or different genes. Complementation testing is a fast and reliable way of determining the number of genetic functions (genes) represented in a large population of randomly isolated conditioned lethal mutants.

Experiment I: Host range of T4 Amber Mutants: Su$^+$ vs Su$^-$ cell types.

| E. coli Strains | Amber Mutants | Gene Effected | Protein Component Coded For | Maturation Step Blocked in Su$^-$ Cells |
|---|---|---|---|---|
| K12 | T4 am B17 | gene 23 | head component | capsid assembly |
| K12(λ) | T4 am N52 | gene 37 | tail fiber component | tail fiber assembly at different steps |
| β | T4 am B252 | gene 35 | tail fiber component | |
| CR63 | T4 am X4E | genes 34, 35, 37 & 38 | all different tail fiber components | |

Materials: PER PAIR OF STUDENTS (for part B only)

| | |
|---|---|
| tryptose-phosphate agar plates | 4 ea |
| PA soft agar tubes each with 3.0 ml | 4 ea |
| pasteur pipettes | 8 ea |
| three inch tubes with .3 ml of phage stock (1 for each phage type) | 4 ea |
| three inch tubes with 1.0 ml of 24 hr cell culture (1 for each cell type) | 4 ea |

Method:

A. Preparation of the phage stocks.

1. Each amber mutant was grown in a log culture of a permissive cell (Su$^+$) type (one of the four listed above).
2. The infected cell culture was vigorously aerated for 6-10 hrs at 30 C to allow for phage replication.

3. Afterwards all the cells were lysed with $CHCl_3$; centrifuge the lysate to remove cell debris and $CHCl_3$. Each lysate contains whole infectious T4 phage particles, all of which still contain the specific amber mutation in their genome.

B. Spot testing 4 cell types with different T4 amber mutants.

1. Preparation of lawn using overlay method. Inoculate a 3.0 ml soft agar tube with 4-5 drops of indicator K12 using a pasteur pipette. Mix and pour onto an agar base plate and label it with strain type. Repeat this for the other 3 strains, let all plates solidify for 15-20 min.

   After overlay of the 4 plates has solidified mark off the bottom of each plate into quadrants. On one plate the 4 different phage stocks will be spotted, label the 4 phage types in the 4 sections found on each plate.

   Using a pasteur pipette put one drop of T4 am X4E in its quadrant on each of the 4 different plates K12, K12 (λ), β, CR63. Repeat this for the other 3 phages using a fresh pasteur pipette each time. Let the spots dry and incubate for 15-18 hours.

   Record the data in a table like table I by using (++) for a spot that causes complete clearing of the lawn; (+) for spot that is not as cleared and (-) for a spot that causes no clearing of lawn. Remember these spots are not plaques, and simply show the presence or absence of phages able to enter productive (lytic) infection in that cell strain. However, in some cases when the spot is very turbid small plaques can be seen. This suggests that the number of phages able to lytically infect that cell type is very small; therefore, confluent lysis in spot is not seen.

TABLE I.

| | amB17 | amB252 | amN52 | amX4E |
|---|---|---|---|---|
| K12 | | | | |
| K12λ | | | | |
| β | | | | |
| CR63 | | | | |

Describe and explain any unusual or significant appearances of the spots (e.g. the appearance of distinct plaques within a cloudy spot). Explain why the T4 amber mutants can cause the clearing of the lawn of one E. coli strain but not another? Be specific. What can you conclude about the genotype (Su$^+$ or Su$^-$) of each of the above strains, i.e. which are permissive and which are nonpermissive?

Questions:

1. Does the presence of a λ prophage effect the ability of K12 to support or not support lytic infection by any of the amber mutants?
2. Which of the above E. coli strains could be the best to use in the preparation of infectious phage stocks of these amber mutants (steps IA)?

Experiment II: In Vitro Complementation Between Cell-free Extracts of Nonpermissive Cell Infected With Two Different Amber Tail Fiber Mutants.

Materials: PER PAIR

| | |
|---|---|
| soft agar tubes (7.5 ml) | 3 ea |
| sterile plates | 3 ea |
| 1 ml pipettes | 4 ea |
| pasteur pipettes | 3 ea |
| 3" sterile tubes | |
| 3" tube with 1.5 ml of T4amB17 extract | 1 ea |
| 3" tube with 1.0 ml of T4amN52 extract | 1 ea |
| 3" tube with 1.0 ml of T4amB252 extract | 1 ea |
| 2.5 ml of E. coli CR63 indicator (see IIA steps 1-6) | |

Method:

A. Preparation for each cell-free extracts of T4amN52, T4amB252 and T4amB17 (Expt. III).

1. Prepare at 37 C two 200 ml* batches of log culture of E. coli β containing approximately $4 \times 10^8$ CFU/ml (in 1 liter flasks). *For T4amB17 need two 200 ml batches and one 100 ml batch of cells. Resuspend the cells in 50 ml instead of 40 ml of buffer.
2. Cool the log culture down to 30 C on a shaker for infection, infect at a MOI of 4 PFU/cell with the desired phage genotype (as prepared in IA) and aerate vigorously at 30 C for 30 min.
3. After 30 min chill the culture in ice water bath to stop all phage replication before the cells lyse.

4. Concentrate the cytoplasm of infected cells by centrifuging it 5,000 × g for 10 min to pellet all the infected cells.

5. Resuspend the cell pellet in 40 ml of buffer (0.039 M $Na_2HPO_4$, 0.022 M $KH_2PO_4$, 0.07 M NaCl, and 0.01 M $MgSO_4$ at pH 7.4) containing 10 μg/ml of DNase.

6. Distribute the resuspended cells into 3" tubes each with 1.0 ml (except T4amB17 each tube should have 1.5 ml). Freeze the cells at -70 C in a dry ice ethanol bath for 15 min, then thaw at room temperature to break open the cells.

   (a) These extracts do not contain fully assembled infectious phage, the phage maturation process has stopped at the step that involves the structural protein coded for by the gene containing the amber mutation.

   (b) The DNase present in the buffer will degrade all cell DNA and all naked phage DNA; therefore, unless the phage DNA has been packaged in the capsid it will be degraded.

   (c) Extracts prepared by method above for T4am B252 and T4am N52 will contain heads with tails (tail-fiberless particles) and will also contain all the tail fiber proteins except the one coded for by the defective gene. Extracts of T4am B17 will not contain any stable assembled heads since this am mutation blocks the synthesis of the capsid protein, thus blocking maturation before phage DNA is surrounded by the capsid.

7. For storage, the extracts may be refrozen at -70 C and stored at -20 C until use in order to maintain the activity.
8. Repeat the above procedures for the other two phages.

B. Cell-free extract complementation between different amber mutant extracts prepared from nonpermissive cells.

1. Since complementation is an all-or-none phenomenon the assay of the extract mixes will be done by a spot test on a lawn of CR63.
2. Preparation of extract mixture.
   - (a) The following combinations of mixtures will be made:

     T4amN52 + T4amB252

     T4amN52 + T4amB17

     T4amB252 + T4amB17
   - (b) The mixtures will be made by the following steps:
     - (1) Pipette 0.5 ml of N52 extract into a clean 3" tube (0.5 ml will be left in original tube which will be used in #3 below).
     - (2) To the clean 3" tube (now with 0.5 ml N52 extract) also add 0.5 ml of B252 extract using a fresh pipette. (The original tube of B252 will have 0.5 ml left in it and it will be used in #4 below.) You now have made N52 + B252 extract mix.
     - (3) Using a fresh pipette add 0.5 ml of B17 extract to the original tube of N52 with 0.5 ml of N52 extract left in it. (You now have made the N52 + B17 extract

mix) and add 0.5 ml of B17 extract to the original B252 tube with 0.5 ml of B252 extract still left.

3. Due to limited materials the controls will not be done. The controls would consist of incubating at 30 C each extract separately for 60 min, then plating them on a lawn of CR63 also.

4. Preparation of indicator lawn for spot test.

   (a) Before the 60 min incubation period is up seed three 7.5 ml soft agar tubes each with 0.5 ml of CR63 indicator. Mix and pour into 3 sterile empty plates. Label one of each of the above mixes made.

   (b) When plates have solidified (30 min) divide the bottom of each plate into halves.

   (c) Using a pasteur pipette place 1 drop of N52-B252 mix on each half of this plate. Repeat for other 2 mixes on other 2 plates using fresh pipettes.

   (d) Allow the drops to dry then incubate 37 C for 15-18 hrs. Record data in the following table, use (++) to indicate the presence of a very clear spot, (+) to indicate a slightly turbid spot, and (-) to indicate no clearing of lawn. The clear spot in the lawn demonstrates that infectious phage are present in the mix.

TABLE II:

| lawn of cell/mixtures | N52 + B252 | N52 + B17 | B252 + B17 |
|---|---|---|---|

Explain the following (be specific):

1. Why was *E. coli* β and not CR63 used to obtain the cell extracts? (see IIA)
2. Why was CR63 and not β used as the indicator in step IIB?
3. What results would you have expected to see on the control plates (IIB2) if they were assayed after 90 min? And Why?

What conclusions can you draw about:

1. The origin of the PFU's detected in the reaction mixtures after 60 min, i.e., how did these infectious phages arise if there were none in the original cell-free extracts (IA6)?
2. The number of genetic functional loci involved? Do the genetic loci that code for proteins 23, 35, and 37 belong within the same gene or are they in separate and distinct genes of the T4 genome? Explain what you know to be true in terms of what experiment II specifically demonstrates.

Questions:

1. Given a series of morphogenetic amber mutants of T4, design an experiment to classify each of them as to the functional components they produce under nonpermissive conditions. That is, how would you determine whether a given mutant synthesized a functional head, functional tail fibers, both, or neither when an $Su^-$ cell was infected with it?
2. In the extract complementation experiment (part IIb), the tail fiber components missing or defective in each of the mutant extracts was effectively replaced in the complementing reaction by an active

component from the other extract. Presumably then, the completed tail fibers combined with tail fiberless particles already present in each extract to yield infectious phage. Design an experiment to determine if tail-fiberless particles from one or both of the extracts were utilized. (Hint: How can you determine if the phage particles isolated from spot of the complementation mixture on the CR63 lawn consists of just one or the other of the parental genotypes or can both parental genotypes be isolated from the same spot?)

3. In part IIB, which of the genotypes are present in the spot of the lawn of CR63 from mix N52 + B17? From mix B252 + B17?

# ENZYME INDUCTION

Living organisms are endowed with a natural constitution of functioning enzymes. These are "constitutive enzymes," which are always present and synthesized by the cells, as distinguished from "inducible" or "adaptive" enzymes which are otherwise not present in appreciable amounts and produced only in times of cell requirements (8).

One of the most carefully studied examples of enzymatic induction is that of β-galactosidase, mainly from the work of Monod and his workers in Escherichia coli (2, 11). Its synthesis is illicited in the presence of lactose, a β-galactoside sugar, and is deemed necessary for the utilization of the sugar. Lactose occurs as a natural substrate for β-galactosidase, serving effectively as a metabolizable energy source and enzyme inducer. This dual relationship has been studied for various other galactosides (15), e.g., several thiomethyl-β-D-galactoside (TMG), an α-galactoside melibiose, and a synthetic sugar isopropyl-thio-β-D-galactoside. These have been observed to act as suitable inducers, but not metabolized by the enzyme.

The introduction of the appropriate inducer will initiate enzyme formation. This reaction, however, may be inhibited by the addition of exogenous energy sources in the form of carbohydrates. Various sugars, e.g., glucose, mennitol, xylose, ribose, fructose, and glycerol have been tested and observed to be effective inhibitors at varying degrees (3, 4, 14). However, glucose has demonstrated to be the most potent and characteristic inhibitor in the carbohydrate group. The persistence of this inhibition varies as a function of concentration of the exogenous energy source. At high concentrations, complete inhibition occurs since the external energy source will be continually utilized in preference to the inducer. At limited concentrations, however, the inhibitory effects are apparent for only a short period, at which time the exogenous source has become exhausted. Recovery commences and induced enzyme synthesis resumes.

Early investigations (3, 4, 12) have shown that it is possible to render cells partially resistant to such inhibitory effects by "pre-induction," i.e., prior exposure of cells to the inducer substance before addition of the exogenous energy source. The degree of resistance depends on the inducer-energy source concentration ratio as well as the length of the pre-induction period.

Bacteriophage infection of a susceptible host cell initiates a chain of events that significantly alters the metabolic machinery of the host, especially the immediate cessation of protein synthesis (10), nearly all bacterial enzymes and mRNA production (5). Phage control directs utilization of the cell pool of nucleotides, amino acids, and some cell enzymes for its own replicative process. In addition, phage infection promptly arrests the induction of repressed host cell enzymes (1). This phenomenon has been extensively investigated for T-even phage infection of Escherichia coli (6, 7), comparing similarity in effects to inducer removal, the nature of enzymatic kinetics, and varying conditions of preinduction.

Early applications of different chemical and physical techniques to measure galactosidase activity provided little satisfactory results. Some interest was developed when the introduction of a chromogenic substrate to measure enzyme activity proved successful. An artificial chromogen, ortonitrophenol-β-D-galactoside (ONPG) had been prepared (16) and first applied by Lederberg (9) to the study of the induction phenomenon in Escherichia coli, based upon coloricmetric determination as a direct measurement of β-galactosidase activity. ONPG represents a colorless chromogenic substrate which when cleaved in the presence of β-galactosidase, liberates o-nitrophenyl, a yellow compound with a measurable absorption peak at 420 mμ. Employment of ONPG have provided a direct, rapid, and specific method for the detction of the β-galactosidase enzyme within a few hours.

# ENZYME INDUCTION

*Cultures*: PER STUDENT

| | |
|---|---|
| *E. coli* $\beta$ (2.5 hours - grown in synthetic M9 medium containing glucose) (centrifuged and dispensed to pellet) | 60 mls |
| Phage stock diluted to $5 \times 10^9$ PFU/ml in synthetic M9 without glucose, T2 | 3 mls |

*Materials*:

| | |
|---|---|
| 0.5% Lactose in synthetic M9 without glucose | 40 mls |
| 0.5% Glycerol in synthetic M9 without glucose | 20 mls |
| Synthetic M9 without glucose | 50 mls |
| ONPG (O-nitrophenylgalactoside) | 9 mls |
| 1*M* $K_2CO_3$ | 25 mls |
| Toluene | 5 mls |
| Demineralized water | 100 mls |
| Sterile screw cap or stoppered tubes | 24 ea |
| Sterile dilution tubes (15 ml volume) | 4 ea |
| 1.0 ml sterile pipettes | 5 ea |
| 10 ml sterile pipettes | 15 ea |
| Spectrophotometer (per class) | 2 ea |
| Blank for spectrophotometer containing 4.5 ml demineralized $H_2O$ | 2 ea |

# ENZYME INDUCTION

Procedures:

1. Each student will resuspend a centrifuge pellet of E. coli β (60 mls of a 2.5 hour culture) with 8 mls of synthetic M9 medium (no glucose). Divide equally and transfer to each of 4 test tubes.

   Label the experimental tubes as follows:

   A = phage infected culture + lactose inducer
   B = normal non-infected culture + lactose inducer
   C = normal non-infected culture + glycerol
   D = normal non-infected culture + no inducer

2. Perform the following for each tube:

Tube A

Resuspended Pellet

↓

Add 1 ml T2 phage

Shake suspension

↓ Wait 2 min

Add another 1 ml T2

Shake.

↓ Wait 2 min.

Bring up to 15 mls with 0.5% lactose

↓

Enzyme Assay

Tube B, C, D (each)

Resuspended Pellet

↓

Add 1 ml of synthetic medium w/o glucose

Shake culture

↓ Wait 2 min

Add second 1 ml synthetic medium w/o glucose

Shake.

↓ Wait 2 min.

| B | C | D |
|---|---|---|
| ↓ | ↓ | ↓ |
| Bring up to 15 mls with 0.5% lactose | Bring up to 15 mls with 0.5% glycerol | Bring up to 15 mls with synthetic medium w/o glucose |
| ↓ | ↓ | ↓ |
| Enzyme Assay | Enzyme Assay | Enzyme Assay |

3. For the enzyme assay, 2 ml samples will be taken from each tube at 30 min intervals (0', 30', 60', 90', 120') and treated as described:

   (a) Add the 2 mls to a tube containing 0.2 mls of toluene. Shake vigorously for 2 min. Incubate in 37 C water bath for 30 min, shaking at 10 min intervals for 1-2 min.

   (b) Remove from 37 C after 30 min and add 0.4 ml of ONPG indicator. Shake. Allow to incubate at room temperature for 15 min.

   (c) Add 1 ml $K_2CO_3$ (1M) to stop the reaction.

   (c) Add 4 mls demineralized $H_2O$. Mix.

   (e) Allow tubes to stand for about 15-30 minutes before transferring an aliquot (~4.5 ml) to a spectrophotometer cuvette for optical density reading at 420 mμ.

   (Be sure to use the same machine throughout the experiment.)

4. Record all data and plot optical density readings as a function of time for each set.

   Compare significant differences observed.

# DNA EXTRACTION

The nucleic acids represent important universal constituents of biological systems. These macromolecules were originally reported by F. Miescher whose work with that of A. Koseel and Hoppe-Seyler provided initial information on class characteristics and component parts of nucleic acids (6). There are two classes of nucleic acids -- deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both types are present in most cell systems, e.g., eukaryotes and bacteria, whereas viral systems generally possess either DNA or RNA.

Successful analyses of nucleic acids depend on the effectiveness of the method in preparation of experimental samples. For example, to obtain DNA several important factors should be considered when choosing a suitable method of preparation: cell breakage, nuclease activity, and mechanical shearing (5a). Insufficient cell breakage fails to release and provide optimal quantities of DNA. Nuclease activity, if not suitably inhibited, results in degradation of the molecule. Some mechanical damage is also apparent, especially shearing of the molecule when handling and transferring the DNA sample.

At present, many of the DNA extraction procedures involve the use of two principal agents, an anionic detergent and a chemical solvent. Employed for the initial extraction step, the detergent serves effectively to release macromolecules from membrane structures as evidenced by the increase in viscosity of the mixture. One of the most commonly used detergents is the sodium salt of dodecyl or lauryl sulfate (SDS), a powerful agent with an apparent ability to inhibit enzyme activity, denature some proteins, and whose negative charge aids in preventing interaction with the nucleic acid (5c). It should be noted that the lithium salt of dodecyl sulfate (LDS) has also been employed occasionally, as well as the more recently introduced amide derivative of sodium dodecyl sarkosinate. In some extractions, lysozyme has been used to aid in cell lysis.

Earlier methods employed chloroform for cell disruption (8). Phenol was found to provide a more efficient reagent for purification of the nucleic acid, i.e., for deproteinization. Phenol treatment, when conducted with a slightly alkaline buffer, will aid in the extraction of DNA from cells with little subsequent RNA contamination (5d). Further purification may be accomplished by treatment with specific ribonucleases which digest RNA and facilitate separation from DNA, and/or pronase, a non-specific, highly active proteinase, to eliminate residual protein contamination. Alternatively, the DNA preparation may be precipitated and washed in ethanol before dissolving in buffer. This procedure may be repeated several times for complete purification.

The various extraction techniques described by different workers (1,2,5d,6) follow these basic procedures with slight modifications of buffer and reagent concentrations: cell disruption with removal of debris and protein by denaturation and centrifugation, followed by purification of the nucleic acid sample. These methods have been applied successfully to isolation of a variety of bacterial DNA's and extended to extraction of phage DNA (1,5e,6). A recent report has described a more convenient and rapid procedure for separation of phage nucleic acid by simply incubating the crude lysate in the presence of a sodium perchlorate solution ($NaClO_4$) at appropriate concentrations of 4-6 M (3,5f). The free nucleic acid is released into the perchlorate within a few minutes and carefully collected. This method has proven successful for the various T-phages and λ of E. coli, some phages of other bacterial species, several RNA phages, and some plant and animal viruses (5f).

A special use of this perchlorate procedure enables a rapid technique for preparation of purified phage DNA from lysates immediately after lysis. This is accomplished by ultracentrifugation of the lysate with perchlorate and sucrose (4). Experiments are still being conducted to determine the details of the mechanism of nucleic acid release, especially of time-concentration dependency, precautions in denaturation, further extraction of other bacterial and animal viruses, and the nature of the DNA product.

It should be considered that an extraction technique proves feasible when it yields a satisfactory product, especially one of a native, highly polymerized state as well as one which is free from protein, lipid, sugar or RNA contamination and free of inorganic salts and other impurities (2).

# DNA EXTRACTION

Cultures: PER STUDENT

*E. coli* β (centrifuge 100 mls of broth culture as a pellet and wash with saline-citrate buffer)

1 ml packed cells (distributed in a 40 ml centrifuge tube)

| Materials: PER STUDENT | Amt. |
|---|---|
| lysing medium (Egg white lysozyme, 4 mg/ml) | 5 mls |
| SDS detergent (1.5% SDS in saline-citrate buffer) | 15 mls |
| saline-citrate buffer (0.15 M NaCl:0.015 M sodium citrate) | 10 mls |
| water-saturated phenol, 80% (preferably distilled) | 15 mls |
| 95% ethanol | 100 mls |
| ether | 5 mls |
| screw-capped 40 ml conical centrifuge tube | 1 ea |
| Pasteur pipette | 1 ea |
| 10 ml pipette | 5 ea |
| stirring glass rods | 10 ea |
| dry ice-ethanol bath | 1 ea |
| ice bath | |
| clinical swing bucket centrifuge | |

Procedures: Extraction of Bacterial DNA

1. Add 4 mls of lysing medium slowly to 1 ml packed cells of *E. coli* β previously washed with saline-citrate buffer and contained in a 40

ml centrifuge tube. Gently mix to resuspend cells and to obtain a smooth suspension.

2. Freeze the mixture in a dry ice-ethanol bath and thaw at 37 C three times.
3. Add 10 mls of SDS detergent, gently stirring continuously for 30 minutes with a glass rod. This will disrupt cells and aid in releasing membrane structures.
4. Add an equal volume of redistilled, water-saturated phenol (80%), about 15 mls. This will give a total volume of 30-35 mls.
5. Gently roll the tube by hand continuously until a smooth, homogenous mixture is obtained (30-45 minutes).
6. Chill the mixture. Centrifuge at 3000 rpm for 15-20 minutes.
7. Collect the aqueous layer carefully with a wide-bore Pasteur pipette (break tip off). This aids in limiting mechanical shearing.
8. Re-extract the phenol-interphase region by addition of 5-10 mls of saline-citrate buffer. Tip the tube back and forth several times. Centrifuge at 3000 rpm for 15 minutes. Collect the aqueous layer and pool with that from the initial extraction.
9. Add 5 mls of ether to the pooled layers to removed the phenol. Gently swirl and allow for separation of layers by chilling in an ice bath. Carefully remove the ether layer (top) with a Pasteur pipette.
10. Residual ether can be removed by bubbling the solution with nitrogen for 15-20 minutes.
11. Selectively precipitate the DNA from the nucleic acid solution by

addition of 2 times the volume with 95% ethanol (50 mls). Add slowly while stirring with a glass rod to collect the fibrous DNA.

12. The DNA product may be suspended in 95% ethanol and stored at 4 C in a covered container until use for base determinations.

## Alternative: Purification of DNA (Marmur method)

**Materials:** PER DNA SAMPLE

| | |
|---|---|
| Dilute saline-citrate buffer | 20 mls |
| Acetate-EDTA | 3 mls |
| Isopropanol | 20 mls |
| Glass stirring rod | 25 mls ea. |

**Procedures:**

1. Drain the spooled nucleic acid sample. Dissolve in 9.0 mls of dilute saline-citrate buffer.
2. Add 1.0 ml of acetate-EDTA. With continually rapid stirring of the solution with a glass rod, add 0.54 volume of isopropanol in a drop-wise fashion. The DNA will selectively precipitate, leaving the RNA and other molecules in solution.
3. Repeat steps 1 and 2.
4. Wash the precipitate in progressively increasing portions of ethyl alchol (70-95%).
5. The sample can be stored in 95% ethanol at 4 C until use.

## Option: Extraction of Phage DNA ($T_4$)

Cultures: PER STUDENT

| | |
|---|---|
| $T_4$ phage ($10^9$-$10^{10}$ PFU per ml) | 100 mls |

Materials:

| | |
|---|---|
| Saline-citrate buffer | 5 mls |
| Water-saturated phenol (88%) | 5 mls |
| capped centrifuge tubes (12 or 15 ml) | 1 ea |
| dialysis bag (Visking No. 2 -- boiled in $Na_2CO_3$ for 10 min.) | 1 ea |
| 500 ml beaker containing saline-citrate buffer | 1 ea |
| ice bath | |
| clinical centrifuge | |

Procedures:

1. Remove debris from crude phage lysate by differential centrifugation two times at 5000 × g (6000 rpm) for 10 min. Pellet phage at 35,000 × g for 30 min.
2. Resuspend phage in saline-citrate buffer at 1/50 of the original volume (2 mls).
3. Titrate and obtain an optical cross-section ($A_{260}$/titer) about $0.7\text{-}1.0 \times 10^{11}$ $cm^2$/PFU.
4. Transfer to a capped centrifuge tube (12 or 15 ml). Mix with an equal volume of saturated phenol and roll in tube for 30 min.
5. Chill the mixture in an ice bath (0-4 C). Centrifuge at 3000 rpm in the clinical centrifuge for 10 min. to facilitate separation of the two phases.

6. Carefully remove the phenol layer (bottom) with a Pasteur pipette and discard. Add a fresh portion of phenol. Repeat this extraction two more times with 10 minute rolling periods.
7. Transfer the aqueous layer to a dialysis bag and dialyze exhaustively against saline-citrate buffer for 24-36 hours. The DNA may be retrieved from the dialysis bag by carefully pouring the sample or with a wide-bore Pasteur pipette.

## BASE DETERMINATIONS

Purine and pyrimidine components may be detected and estimated as free bases. They can usually be released from nucleic acid samples by hydrolysis with different acids, e.g., hydrochloric, perchloric, or formic acids. The resulting base hydrolysates can be subjected to the various methods that have been developed for the separation and quantitative analysis of nucleic acids and for the identification of their components. One of the most feasible techniques generally employed to study components, especially of the purine and pyrimidine bases, is the application of paper chromatography, first demonstrated by Vischer and Chargaff (10) and Hotchkiss (6). Successful resolution of components depends on several factors, especially sensitivity to varying environmental conditions (3). For example, the rates of migration and final separation of the substances are particularly influenced by the composition of the solvent, the pH, temperature, water and ion concentrations in the developing system. Qualitative resolution is also affected by the type of filter paper employed, as differences in paper grades will reflect varying mobilities in the same solvent system. Interference by metal ions or UV-adsorbing materials in the paper may result in streaking and present an erroneous band profile. Equally important is the application of sufficient amounts of the sample for analysis. Earlier studies required larger quantities, usually milligrams, for effective results. However, special refinements in techniques (4) have enabled analysis of nucleic acids in microamounts.

Detection on the paper chromatograms may be accomplished by several methods, e.g., chemical treatment by conversion of the base to suitable salts (8) or treatment with mercury to form complexes detectable with dyes of fluorescence. However, the more general and convenient technique utilizes the high and specific characteristic adsorption intensity for ultraviolet light by the purine and pyrimidine bases, which under UV light will appear as visible dark regions or shadows against the filter paper background. The separated components are located and computed in terms of Rf values which reflect the rate of migration and relative position of each substance. These may then be eluted from the paper chromatograms for recovery purposes or quantitative measurements by spectrophotometry (5a).

Recent application of thin-layer chromatography has provided another effective and sensitive means to analyze nucleic acid components (5b). These developments mainly involve the use of different types of thin-layers suitable for efficient separation of the various bases and other compounds (5b). There also have been some reports of the use of electrophoretic methods for base determinations (2,3). Quantitative assays by the elution technique as well as absorption determinations may be conducted in a fashion similar to those described for paper chromatography.

Various refined methods are also available for further studies of nucleic acids, especially for physical characterization. These techniques provide separation and purification of various classes of

DNA or RNA based on elution by charge and/or size of the molecules through different types of columns, e.g., MAK (5c), nitrocellulose (5c), or hydroxyapatitie--an inorganic, crystalline, insoluble salt (1,7)--columns. Buoyant density methods have been applied with zone sedimentation in sucrose gradients or sedimentation equilibrium in cesium chloride.

The introduction and application of these various chromatographic methods provided initial progress for nucleic acid research. It led to the recognition of the composition profile of nucleic acids from a variety of sources. Developments in techniques have allowed quantitative separation and estimation of individual components with respect to purine and pyrimidine bases and other related substances. Adaptation of refined techniques in elution columns and ion-exchange columns have led to investigations on physical characterization and presented important knowledge of the molecular structure of nucleic acids.

# BASE DETERMINATIONS

## Chromatographic Determinations:

### Materials:

| | |
|---|---|
| Hydrolyzed DNA sample of each student as prepared by the lab instructor | 0.1 ml |

PER GROUP OF STUDENTS (3):

| | |
|---|---|
| Control samples of individual bases--adenine, guanine, thymine, cytosine, 5-hydroxymethylcytosine (5-HMC), uracil | 0.1 ml ea |
| Control sample of mixture of all the bases | 0.1 ml |
| Chromatographic filter paper (Whatman no. 1) | 1 sheet |
| Solvent system in large developing tank or jars-- | |
| Isopropanol-HCl solvent | 1 |
| Isopropanol-$NH_3$ solvent | 1 |
| Lambda pipettes (50 lambda)--if not available, substitute with Pasteur pipettes | |
| Hand dryer | |
| Scissors | |
| Cellophane paper | |
| Glass plates | |
| Ultra-violet light source (lamp or hand light) | |

### Procedures:

DNA samples will be provided as base hydrolysates prepared by acid hydrolysis with formic acid prior to the lab exercise by the lab instructor.

Students will work in groups of three in preparation of paper chromatograms and spotting of samples for analysis.

1. Each group of students should cut a sheet of Whatman no. 1 filter paper (18" x 22") in half to provide duplicate sheets (18" x 11"). Be careful to avoid frequent handling of the filter paper except along the edges. Fingerprints may interfere with separation of components and subsequent band profile.
2. Spot 40-50 lambda of each control sample and the control base mixture approximately 1.5 inches from the bottom of the 11" side, making each spot 1 inch apart. Keep the spots about 1 to 1.5 centimeters in diameter. Apply a small portion of the sample initially; dry with the hand dryer. Then continue spotting and drying until the appropriate amount of the sample has been applied.
3. Each student will then in turn spot his own hydrolysate (40-50 lambda) in a similar fashion as the controls, repeating steps 2 and 3. Duplicate chromatograms will be prepared. At the top of each, identify each sample and the type of developing solvent used.
4. The one-dimensional ascending technique will be employed. When all the sample have been dried, carefully join the two ends of each paper together (18" side) to form a cylinder. Secure with staples.
5. Carefully place one chromatogram in the isopropanol-HCl system and the duplicate sheet in the isopropanol-$NH_3$ system with the

papers in standing position.

6. Allow the chromatograms to develop 18-24 hours. Remove and mark the solvent front with pencil. Hang the papers to dry.
7. Using an ultra-violet light source, locate the separated components. Outline each spot with pencil on the paper itself.
8. Determine and record the Rf value for each spot, using this formula:

$$Rf = \frac{\text{distance of spot from point of application (cm)}}{\text{distance of solvent front from point of application (cm)}}$$

Compare your values with those given on the attached table.

9. If the components are well separated and distinguishable, the chromatograms may be employed for quantitative assay of each component by spectrophotometry.

TABLE. Rf Values for Purine and Pyrimidine Bases*

| Base | Solvent System<br>Isopropanol-HCl | <br>Isopropanol-$NH_3$ |
|---|---|---|
| Adenine | 0.37 | 0.32 |
| Guanine | 0.16 | 0.22 |
| Cytosine | 0.22 | 0.44 |
| Thymine | 0.52 | 0.76 |
| Uracil | 0.38 | 0.66 |
| 5-Hydroxymethylcytosine | 0.25 | 0.44 |

*E. Chargaff and J. Davidson, The Nucleic Acids, Vol. I, Chapter 7, Academic Press, 1955.

## Option: Spectrophotometric Determinations

Sometime prior to the experiment, each group of students should carefully cut out each outlined spot from the chromatograms. Shred each disc sample and transfer to individual test tubes. Be sure to properly label each sample and the developing solvent system used.

Submit all test tubes to the lab instructor who will prepare them for extraction with HCl. Samples will be eluted and ready for centrifugation and optical density determinations by students during the laboratory period.

### Materials:

| | |
|---|---|
| Eluates of each spot component submitted by students | |
| Centrifuge tubes (12 ml or 15 ml) | 1 per sample |
| Clean test tubes | 1 per sample |
| Spectrophotometer cuvettes | |

### Procedures:

1. Decant the eluate into clean centrifuge tubes and centrifuge at 3500 rpm for 15 minutes to pellet lint and other debris.
2. Save the supernatant portion by decanting into clean test tubes.
3. Transfer each sample to spectrophotometer cuvettes and measure at the adsorption maximum (260 mμ) using a Beckman DU spectrophotometer. Be sure to include a corresponding blank solution at the same adsorption maximum.

# ANIMAL VIRUSES: IN VITRO STUDIES

Cell culture research has proven to be an extremely useful tool in virology, especially in providing cell systems to study various aspects of host-virus interactions in vitro--the influence of cell growth stage and type, virus replication, and progeny maturation, the synthesis and assay of interferon, and other indices of biological activity.

Mammalian cells exhibit greater complexity in nutrient requirements than do simple organisms such as bacteria. The diversity of cell types presents various problems in approaches to their cultivation and propagation in vitro. It can be expected that fluctuations in environmental conditions, e.g., temperature, pH, oxygen and carbon dioxide tension, redox potentials will affect significantly the growth and metabolism of different cells. Nutritional requirements including inorganic ion concentration, energy source, amino acids, serum protein concentration as well as the presence of specific growth factors will vary from one cell line to another. In addition, contamination presents a continuing problem. With the introduction of antibiotics, there is some apparent influence on cell multiplication and proliferation by reason of some degree of cell toxicity.

One special characteristic of almost all cell culture systems is that the most nearly ideal media required for growth and maintenance invariably contain serum. Serum is known to contain a variety of proteins, enzymes, natural antibodies, a few cytotoxic substances, and some growth factors whose nature still remains obscure. Different investigations have been conducted to study the effects of serum-free media on the nutrition and metabolism of various cell types (2a,2b,12). These studies have indicated that comparable growth rates and cell yields can be obtained with serum-free and with serum-containing media. Such observations, however, are limited to the cell types employed and further investigations will be necessary before that can become a general evaluation applied to all cell lines. A variety of chemically-defined media have been developed and shown to support growth of different cell lines in suspension systems (6,7,8).

The various types of serum-free media described differ from one another in the addition of a component observed to be responsible for growth stimulatory activity, e.g., lactalbumin hydrolysate, insulin (2a, 3), or different serum fractions such as serum albumin (4,9,10,14,15). Some studies have employed Bacto-Peptone, a heat-stable enzymatic digest prepared from animal tissue, as a serum substitute (5). A recent report conducted a biological titration of whole peptone and fractionated peptone dialysates to look for stimulation of cell growth. Results indicated that the different fractions isolated supported growth, but to varying degrees (11).

With the introduction of growth of cells in serum-free media, studies can be extended to determining the competence of such cultures to support virus replication. Little work had been done in this area except for a report by Walker et al. (16). A recent study has been conducted monitoring virus growth and yields of Venezuelan equine encephalomyelitis virus in serum-free and chemically-defined medium in comparison to growth in serum-containing medium. Results indicate virus multiplication in serum-free medium gives comparable yields (13). A recent study examines the role of cell nutrition in enhancing virus replication (1). The data presented show that nutritional factors selectively added to cultures, and thus identifiable, played an important role in virus replication.

Virus titer in animal cell culture often is determined by the plaque assay method, similar to that conducted for bacteriophage titrations on a bacterial host. The method in tissue culture involves infection or adsorption of the virus to a cell monolayer (sheet of susceptible animal cells). A nutrient agar is placed over the cells and later stained with a vital dye to delineate virus plaques. This technique was demonstrated to be a precise method for measuring cell culture infectivity of several animal viruses (1). Various modifications have been made since Dulbecco's 1952 report, including the use of different overlays as well as additional supplements that may influence or enhance plaque formation.

Agar has remained the best solidifying agent and is generally accepted for routine titration of animal viruses. The availability of alternative overlays has been investigated. An early study reported the use of methylcellulose gel as an agar substitute (2). Although it appears less efficient, it does provide an alternative to agar for special uses and is not toxic to animal cell tissue. Employment of this agent has been applied to a recently introduced plaque assay system in the absence of a $CO_2$ atmosphere using a bicarbonate-free overlay (3,4). Generally, animal cells in culture are incubated in a $CO_2$ atmosphere. Results of these studies indicate an improvement in plaque and virus yields with methylcellulose agar over those obtained with Bacto-agar. The method has been employed successfully for the titration of a number of animal viruses.

## IN VITRO STUDIES

### I. Preparation of Cell Cultures: Chick Embryo Monolayers

**Materials:** PER PAIR OF STUDENTS

| | |
|---|---|
| 10-day incubated chick eggs | 8 |
| 95% ethanol in beaker | 1 |
| Straight forceps (or egg cracker) | 1 |
| Curved forceps | 1 |
| 60 mm plastic tissue culture plates | 1 |
| 125 ml sterile flask with magnetic stirring bar | 2 |
| 125 ml sterile flask | 2 |
| 50 ml sterile syringe | 1 |
| Centrifuge tubes, 40 ml | 1 |
| Funnel with gauze layers | 1 |
| sterile 10 ml pipette | 10 |
| sterile 1.0 ml pipette | 5 |
| ice bath | |

**Media and Solutions:**

| | |
|---|---|
| 1× Eagle's medium, Nagle's medium | 150 mls ea |
| 0.125% trypsin | 50 mls |
| Phosphate buffered saline (PBS) | 100 mls |
| 1× Hanks' balanced salt solution | 100 mls |

Procedures:

NOTE: It is important to maintain equipment sterility and carry out manipulations under aseptic conditions.

1. Wash each incubated candled egg by dipping in 95% ethanol. Allow the eggs to air dry by standing eggs with air sac up. With a sterile egg cracker or pair of sterile straight forceps (dipped in alcohol and flamed), carefully break the eggs above the air sac and remove all egg shell.
2. Using sterile curved forceps, remove the chick embryos from each egg by the neck and place in a sterile petri dish. Decapitate. Remove embryo bodies to a 125 ml sterile flask. Wash 2 times with warm 1× Hanks' solution (37 C), 50 ml portions. Carefully pour off the wash each time, flaming the flask mouth.
3. Remove the embryos into a 50 ml sterile syringe and mince into another 125 ml sterile flask. Wash the tissue twice with warm PBS, 50 ml portions. Decant carefully, saving minced embryo.
4. Add 3 mls of warm trypsin (37 C) per embryo to the mince. Place on magnetic stirrer and trypsinize vigorously for 20 minutes. Prop the flask to allow the tissue to settle. Gently pour the supernatant fluid into a 125 ml flask containing 50 mls of complete Eagle's medium. Keep on ice.
5. Repeat trypsinization for remaining tissue, adding 2 mls of trypsin per embryo, for 10 minutes. Allow tissue to settle. Add supernatant cells to the initial harvest.

6. Filter the harvested chilled mixture through the gauze layered funnel into a centrifuge bottle. Centrifuge at 2500 rpm for 10 minutes (International centrifuge).
7. Decant and discard supernatant. Resuspend cell pellet into 20 ml Eagle's medium by pipetting cells up and down several times. Transfer to sterile 40 ml centrifuge tube. Centrifuge at 1500 rpm for 10 minutes. Note the quantity of packed cells.
8. Plates will be prepared in sterile 60 mm plastic tissue culture dishes, adding 1 ml packed cells to each 160 mls of Eagle's medium or 160 ml of complete Nagle's medium. Resuspend cells in medium uniformly before addition to the media. Place on stirrer to mix.
9. Seed each plate with 5 mls of the cell suspension with a sterile 10 ml pipette.
10. Gently mix by rotating plates several times back and forth. Incubate at 37 C in a $CO_2$ atmosphere for 42-48 hours. Observe for formation of cell sheet or monolayer, checking at 4, 8, 16, 24, 36 and 48 hours.

See Figures 4-7, demonstrating cell monolayer formation during a 24 hr growth period.

Figure 4. Growth of chick embryo fibroblast cells at t= 0 hrs.

Figure 5. Growth of chick embryo fibroblast cells at t = 3 hours.

Figure 6. Growth of chick embryo fibroblast cells at t = 7 hours.

Figure 7. Growth of chick embryo fibroblast cells at t = 24 hrs.

## II. Plaque Assay with Sindbis Virus (or with Newcastle Disease Virus)

Cultures: PER PAIR OF STUDENTS

| | |
|---|---|
| 18-24 hours chick monolayers | 14 plates |
| Sindbis virus, LP and SP ($10^5$ PFU/ml) | 1 ml ea |
| NDV ($10^5$ PFU/ml) | 1 ml ea |

Materials:

| | |
|---|---|
| 1× Hanks' solution | 50 ml |
| sterile dilution tubes | 8 |
| sterile 10 ml pipette | 10 |
| sterile 1.0 ml pipette | 20 |
| agar-medium overlay (Mix 40 ml of 2× Eagle's medium with 40 ml of 2% Bacto-agar and maintain at 45 C until use) | 80 mls |

Procedures:

Each pair of students will titrate a LP and SP virus stock.

1. Pipette or aspirate off the medium from each plate.
2. Dilute the virus stocks to the $10^{-4}$ dilution. Plate 0.2 mls of $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions, using 2 replicate plates per dilution. Allow 2 cell controls containing no virus, only Hanks' diluent.
3. Rock plates back and forth several times at 15 minute intervals. Adsorb for 45-60 minutes.
4. Gently, with a sterile 10 ml pipette overlay each plate with 4 mls of the agar-medium mixture per plate. Allow agar to solidify.

Invert and incubate at 37 C in a $CO_2$ atmosphere for 2 days. Stain with neutral red. Count plaques and determine the virus titer. Observe any differences in plaque morphology.

See FIGURES 8 and 9 for the characteristic plaque size.

Figure 8. Large Plaque Sindbis on Chick Embryo Fibroblast Monolayers.

Figure 9. Small Plaque Sindbis on Chick Embryo Fibroblast Monolayers.

## *IN VIVO* STUDIES

Sindbis virus is a group A arbovirus (togavirus) that causes acute encephalitis in newborn mice. The disease, however, does not progress to death in young adult mice (1). These observations provide the basis for an *in vivo* study. Students will be able to observe the pattern of developing resistance as a function of age, to monitor virus dissemination through various tissues, and to determine interferon and antibody levels. The results of such studies were reported in detail in a recent paper investigating age-dependent resistance (2).

A second study has also been presented extending investigations into the histopathological changes that occur in subclinical infection of young adult mice (1). The authors have included some *in vitro* studies by establishing cells derived from newborn and adult mouse tissue in cultures and determining whether these exhibit susceptibility to Sindbis infection similar to that seen *in vivo*.

# IN VIVO STUDIES

## A. Inoculation of Mice

Animals: PER GROUP OF 4 STUDENTS

| | |
|---|---|
| Newborn mice (1-3 days old), with mothers | 12 |
| Weanling mice (20-25 days old) | 18 |
| Large plaque Sindbis virus ($10^4$ PFU/ml) | 4 mls |

Materials:

| | |
|---|---|
| sterile disposable 1 cc tuberculin syringe, needle | 4 ea |
| 95% ethanol in a beaker | |
| cotton | |
| ether | |
| brown paper | |
| brown tape | |
| ice bath | |

Procedures:

Students will work in groups of 4 students, two students with the group of newborn mice and two with the group of weanling mice. The data will be compiled and computed by all the students. All students should set up a working area for inoculation of the mice. Tape down each corner of a sizable piece of brown paper. Have cotton, alcohol, and ether on hand (CAUTION: Turn off all Bunsen burners!!) Keep the virus stock in an ice bath during the inoculation procedures.

1. The first pair of students will work with 12 newborn mice, dividing into groups of 6 mice each, one set to be inoculated intracerebrally and the second set intraperitoneally.
2. With a sterile 1 cc tuberculin syringe, draw up 1 ml of the virus stock and replace securely into the syringe holder until use for inoculation. Tape the syringe to the brown paper.
3. Apply ether to the cotton and anesthetize one mouse. Swab the area with alcohol and inoculate with 0.05 ml of the virus stock. Proper injection procedures will be demonstrated by the lab instructor. Avoid as much leakage of the inoculum as possible.
4. Continue injections until all mice in each group have been inoculated. Each student will then divide his 6 mice into groups of 3 mice each to be placed in two different cages. One set will be used for obtaining the virus harvest from tissues in two days. The second group will be used to observe for mortality.
5. Label each cage of mice:

   NAME
   AGE OF MICE
   ROUTE OF INOCULATION (IC or IP)
   DATE OF INOCULATION

6. Check daily for the rate of mortality and record the date of death. Remove and dispose of dead mice during this observation period (1-2 weeks).
7. The second pair of students will work with 18 weanling mice, separating into groups of 9 mice each, one set inoculated IC and the second set IP. Follow the same procedures as described

in steps 2-6. Each students, however, will divide his 9 mice into groups of 6 mice and 3 mice to be placed in two different cages. Two days post-inoculation, 3 of the 6 mice will be sacrificed and harvested for virus. The remaining 3 mice will be sacrificed on day 5 post inoculation. The second group of 3 mice will be observed for rate of mortality. Maintain observations for 1-2 weeks.

## B. Harvesting Organs from Mice

Materials: PER STUDENT

| | |
|---|---|
| one pair or large scissors | 1 ea |
| one pair of small scissors | 1 ea |
| 95% ethanol | |
| plastic petri dish | |
| centrifuge tube (12 or 15 ml) | |

Procedures:

1. On the second day post-inoculation, students will sacrifice the newborn gorup of mice and harvest from each the following: brain, serum, spleen, and liver. On the second and fifth day post-inoculation, students will harvest the weanlings.
2. The dissection procedures will be described and demonstrated in detail by the laboratory instructor. Set up the working area and materials as before. Materials will be sterilized by dipping in alcohol and flaming.

3. Decapitate each newborn mouse and immediately collect the blood. Pool from all 3 mice. Place the blood in the refrigerator overnight to clot. Loosen the clot from the tube wall and centrifuge at low speed to sediment RBC. Transfer the supernatant serum to a vial and freeze until assayed.
4. The organs from each mouse will be obtained by dissection and placed in one plastic petri dish kept chilled in an ice bath. This aids in avoiding dehydration of organs, especially of the mouse brain. To dissect, pour alcohol over the area of dissection. Allow to drain and proceed to dissect as described by the instructor. Repeat procedures for each mouse. Immediately freeze all organs samples until assay.
5. When completed, wrap the animal carcasses in the brown paper. This will be disposed of by incineration.
6. Be sure to properly label each dish:

    AGE OF MICE
    ROUTE OF INOCULATION
    ORGAN
    DAY OF HARVEST
    TITRATION SET 1, 2 or 3

## C. Preparation of Tissue Homogenates for Virus Assay

Materials: PER STUDENT

| | |
|---|---|
| Organ samples from each mouse group | |
| mortar and pestle, chilled and sterile | 1 per organ |
| sterile centrifuge tube | 1 per organ<br>(for weanlings) |

| | |
|---|---|
| sterile tubes for centrifugation (to contain less than 1 ml volume) | 1 per organ (for newborns) |
| sterile PBS | |
| sterile fine glass beads | |
| sterile forceps | |
| sterile test tubes | 1 per organ |
| sterile 1.0 ml pipettes | |
| sterile 10 ml pipettes | |

Procedures:

Each set of organs will represent one titration.

1. Transfer all plates to an ice bath. Set up a second ice bath to chill the sterile mortar and pestles. It is best to do one plate of organs at a time. First determine the weight of each organ by weighing the entire plate. Transfer one organ to a sterile mortar and pestle. Reweigh the plate. The difference in the two weights represents the weight of the removed organ. Repeat for the remaining organs. Record all weights.
2. Add a very small amount of sterile fine glass beads to each organ. Homogenize the tissue. Then add sterile PBS. The amount (in mls) is obtained by multiplying by nine the weight of the organ. Homogenize again to obtain a uniform tissue homogenate. Carefully transfer to a tube and centrifuge at low speed for 10 minutes. Collect the supernatant fluid for assay.

Continue with the second set of organs and, then the third set of organs as described in steps 1-2.

## D. Assay of Tissue Homogenate Samples

Materials: PER GROUP OF 4 STUDENTS

| | |
|---|---|
| Chick embryo monolayer cultures (18-24 hours), prepared earlier | 48 for newborn<br>96 for weanling |
| tissue homogenate samples | |
| sterile PBS | 700 mls |
| sterile dilution tubes | 50 for newborn<br>65 for weanling |
| sterile 1.0 ml pipettes | 120 |
| sterile 10 ml pipettes | 10 |
| ice bath | |
| agar-medium overlay (2× Eagle's medium with 2% Bacto-agar), keep at 45 C when mixed | 500 mls for entire assay |

Procedures:

1. This will be conducted as a routine plaque assay. Aspirate or pipette off the medium from each plate.
2. Inoculate plates with 0.2 ml of homogenate sample dilutions. Adsorb 45-60 minutes at 37 C. Rock plates at 15 minute intervals.
3. Overlay each plate with 4 mls of the agar-medium mixture. Allow to solidify. Invert and incubate under $CO_2$ for 2 days. Stain. Count plaques. Record as the number of PFU per mg of tissue.
4. These are suggested dilutions to use when plating for the various samples from each group of mice:

| | | |
|---|---|---|
| Newborn -- 2 day harvest | Brain | -4,-5,-6 |
| IC and IP | Serum | -2,-4,-6 |
| | Spleen | -3,-4,-5 |
| | Liver | -4,-5,-6 |
| Weanling -- 2 day harvest | Brain | -3,-4,-5 |
| IC and IP | Serum | -3,-4,-5 |
| | Spleen | -2,-3,-4 |
| | Liver | -2,-3,-4 |
| Weanling -- 5 day harvest | Brain | -2,-3,-4 |
| IC and IP | Serum | -2,-3,-4 |
| | Spleen | und,-1,-2 |
| | Liver | und,-1,-2 |

5. Evaluate the data obtained and make comparisons in the two sets of mice as a function of host age, route of inoculation, mortality, and comment on virus dissemination to the various organs.

# APPENDIX I

## Results, Discussion, and Experimental Data

## PHAGE ASSAYS

### Discussion, Results

In phage assays, the various factors that contribute to the growth and development of phage particles have already been described. A few of these factors, e.g., the state and age of the host indicator, the incubation period for plaque development, and nutritional requirements have been investigated and compared under varying conditions.

### a. Physiological state of the indicator host

Cultures of E. coli β grown in an enriched nutrient medium, tryptose phosphate broth ($TPO_4$), and in a synthetic medium of essential salts and one carbon source (M9) indicated differences in cell concentrations when assayed for viable counts at 6 and 24 hours. Optical densities were also observed. In TABLE I it was observed that cell densities and concentrations were reproducible and comparable in the $TPO_4$ cultures. However, M9 cultures gave low optical densities and viable counts were not prepared. This situation was attributable to the longer lag period required by cells for growth in synthetic media (to be discussed in "Unrestricted Cell Growth" experiment).

Since the $TPO_4$ cultures demonstrated reproducible cell numbers, an E. coli β culture grown either for 6 or for 24 hours was employed as indicator host for the assay of phage T1 and compared. The results in TABLE Ia indicated that the young culture may be used as an effective indicator as the older culture and still provide comparable results. It is important, however, to remember that cultures will vary from preparation to preparation (TABLE 1), and that bacterial growth is influenced by a variety of factors. Such variations are attributable to the quality of the medium, the size of the initial inoculum, the amount and rate of aeration, the optimal incubation period, the volume of media used, as well as the surface area of the incubating flask.

### b. Plaque development

Similar factors also affect the ultimate size, appearance, and numbers of phage plaques. The appearance of the first plaques may be detected within several hours after incubation. This may represent initial numbers or final maximal numbers for different phages. When plaque development has been completed, it can be expected that bacterial hosts and phage reinfection of susceptible cells have ceased further growth, and most of the nutrients in the medium have already been exhausted. The characteristic plaque size and morphology for the particular phage is seen then.

Employing the conventional soft agar overlay method for assay, plaque counts for T1 and T2 were compared after 5 hours and 24 hours of incubation at 37 C (TABLE II). By 5 hours, little more than 50% of plaque development has been completed for T2. For T1, however, development appeared essentially completed and comparable to the 24 hour determination. Maximal titers in most cases can be expected to have been reached by at least 15 hours of incubation.

In phage assays, the techniques employed must prove to be so efficient and precise, that results are highly reproducible. Various methods and their modifications have already been presented and will be evaluated on this basis. The conventional soft agar overlay technique has been observed to be the most useful and accurate method for phage assays and will serve as the comparative base for three other methods to be described: the agar overlay without basal layers, the limiting-end point dilution method, and the spread technique.

Comparisons of phage assays for T1 and T2 with basal agar and without basal agar layers are presented in TABLE III. Varying amounts of soft agar overlay in the absence of basal agar were used and observations made as to plaque visibility, size, and numbers. It appears that smaller volumes of soft agar (2.5 or 3.0 ml) were less suitable, the indicator lawn being a sparser growth, thereby presenting fewer visible plaques and creating counting difficulty. The larger volumes, especially the 5.0 and 7.5 ml, seemed more suitable and effective assay volumes. Plaque sizes were variable in all cases. The phage titers determined for T1 with these volumes appeared fairly comparable (mean and standard deviation) to those obtained with basal layers. However, for T2, a correction factor of two x, as described earlier (Mora, 1963), must be applied in order to arrive at a more accurate value.

A second method involves the spread plate technique in which the phage-infected culture is inoculated directly onto the surface of a basal agar plate and spread evenly with a glass L-rod. TABLE V indicates that results obtained in T1 and T2 assays were comparable to those by the conventional overlay method.

The limiting-end point dilution method was also used to determine phage titers, as evidenced by the presence of lysis of varying dilutions of phage-infected cultures. The last dilution indicating complete lysis of the indicator cells represented the phage titer, hence, the limiting-end point. TABLE IV gives the readings obtained for T1 and T2 at 24 and 48 hours. Complete lysis could be seen for the first few dilutions by 24 hours, but with varying degrees of lysis for the remaining dilutions. By 48 hours there is less evidence of complete lysis, as the development of phage-resistant cells have gradually increased to significant numbers. The phage titer determined is less precise and expressed only as the reciprocal of that dilution showing complete lysis. Results seem less reproducible and the method does not appear as effective as the conventional overlay method for assay.

In conclusion, phage samples may be titrated successfully by the several methods described -- the conventional soft agar overlay, soft agar overlay without supporting basal layers, or the spread plate technique -- with simple procedures and comparable results. The limiting-end point dilution method, however, is restricted and insignificant if less than half of the dilutions indicate complete lysis, and is, therefore, applicable to certain limited experiments.

TABLE I. *E. coli* β indicator grown in tryptose phosphate and synthetic M9 media

| Medium | Incubation Time | Optical Density | No. bacteria per ml |
|---|---|---|---|
| Tryptose phosphate | 6 hr | 0.3 | $4.7 \times 10^8$ |
| | | 1.7 | $7.0 \times 10^9$ |
| | 24 hr | 1.7 | $7.0 \times 10^9$ |
| | | 1.6 | $1.6 \times 10^9$ |
| Synthetic M9 | 6 hr | - | - |
| | 24 hr | 1.0 | $6.5 \times 10^8$ |
| | | 1.8 | $2.8 \times 10^9$ |

TABLE Ia. Phage assay of T1 employing a 6 hour and 24 hour E. coli β indicator

| Age of culture | Number of PFU | PFU per ml |
|---|---|---|
| 6 hrs | 37 | 3.2 × $10^9$ |
| | 26 | |
| | 39 | |
| | 34 | |
| 24 hrs | 35 | 3.1 × $10^9$ |
| | 30 | |
| | 30 | |
| | 29 | |

TABLE II. Plaque development for T1 and T2

| Phage | Sample | Incubation Period | PFU per ml |
|---|---|---|---|
| T1 | 1 | 5 hr. | $7.0 \times 10^{9}$ |
| | | 24 hr. | $7.5 \times 10^{9}$ |
| | 2 | 5 hr. | $2.9 \times 10^{10}$ |
| | | 24 hr. | $3.1 \times 10^{10}$ |
| T2 | 1 | 5 hr. | $1.1 \times 10^{10}$ |
| | | 24 hr. | $2.0 \times 10^{10}$ |
| | 2 | 5 hr. | $1.3 \times 10^{10}$ |
| | | 24 hr. | $2.1 \times 10^{10}$ |

TABLE III. Comparisons of phage assay with basal agar plates and without basal agar layers

| Phage | Amount of Overlay | PFU per ml | Mean | s | Plaque size (mm) |
|---|---|---|---|---|---|
| T1 | control[c] | $4.0 \times 10^9$ | 121.4 | 18.4 | 3.82 |
| | 2.5 ml | $2.8 \times 10^9$ | 158.4 | 17.5 | 1.88 |
| | 3.0 ml | $3.1 \times 10^9$ | 172.0 | 12.2 | 2.02 |
| | 5.0 ml | $3.2 \times 10^9$ | 155.2 | 15.3 | 2.33 |
| | 7.5 ml | $2.9 \times 10^9$ | 129.6 | 24.3 | 2.88 |
| | 10.0 ml | $2.4 \times 10^9$ | 103.2 | 13.1 | 2.85 |
| T2 | control[c] | $1.2 \times 10^{10}$ | 121.4[a] | 4.3[b] | 0.267 |
| | 2.5 ml | $8.0 \times 10^9$ | 158.4 | 10.5 | 0.183 |
| | 3.0 ml | $8.5 \times 10^9$ | 172.0 | 10.7 | 0.235 |
| | 5.0 ml | $8.0 \times 10^9$ | 155.2 | 10.6 | 0.307 |
| | 7.5 ml | $6.5 \times 10^9$ | 129.6 | 7.3 | 0.394 |
| | 10.0 ml | $5.0 \times 10^9$ | 103.2 | 9.9 | 0.398 |

[a] obtained by correction factor of two times the PFU values

[b] determined by using the above means

[c] 2.5 ml overlay plus 20 ml basal agar

TABLE IV. Assay Methods -- Limiting-end point dilution and soft agar layer method

| Phage | Incubation Time | Tube Number | | | | | | | | | | PFU per ml |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| T1 | 24 hr. | 0 | 0 | 0 | 0 | 0 | 0 | +3 | +4 | +4 | +4 | $10^6$ |
| | | 0 | +2 | 0 | 0 | 0 | +2 | +3 | +4 | +4 | +4 | |
| | | 0 | 0 | 0 | 0 | 0 | +3 | +4 | +4 | +4 | +4 | |
| | | 0 | 0 | 0 | 0 | +3 | 0 | +3 | +4 | +4 | +4 | |
| | 48 hr. | 0 | +2 | 0 | 0 | 0 | 0 | +3 | +4 | +4 | +4 | |
| | | 0 | +2 | 0 | 0 | 0 | +2 | +3 | +4 | +4 | +4 | |
| | | 0 | 0 | 0 | 0 | 0 | +2 | +3 | +4 | +4 | +4 | |
| | | 0 | ± | ± | 0 | +2 | ± | +3 | +4 | +4 | +4 | |
| | 24 hr. (soft agar) | | | | | | | | | | | $2.64 \times 10^7$ |
| T2 | 24 hr. | 0 | 0 | 0 | 0 | +2 | +1 | ± | +2 | ± | +4 | $10^7$ |
| | | 0 | 0 | 0 | ± | +1 | +2 | ± | +1 | ± | +4 | |
| | | 0 | 0 | 0 | 0 | +1 | +1 | ± | ± | +1 | +4 | |
| | | 0 | 0 | 0 | ± | +2 | +2 | +1 | +1 | +2 | +4 | |
| | 48 hr. | 0 | 0 | +2 | +1 | +2 | +2 | +2 | +2 | +1 | +4 | |
| | | 0 | 0 | +1 | +2 | +2 | +2 | +2 | +2 | +1 | +4 | |
| | | 0 | 0 | ± | +1 | +2 | +2 | +2 | +2 | +2 | +4 | |
| | | 0 | 0 | +1 | +2 | +2 | +2 | +2 | +2 | +3 | +4 | |
| | 24 hr. (soft agar) | | | | | | | | | | | $9.8 \times 10^7$ |

TABLE V. Assay methods -- spread plate and soft agar techniques

| Phage | Method | PFU per ml |
|---|---|---|
| T1 | soft agar | $3.1 \times 10^{9}$ |
| | spread plate | $3.8 \times 10^{9}$ |
| T2 | soft agar | $3.12 \times 10^{10}$ |
| | spread plate | $1.94 \times 10^{10}$ |

# STATISTICAL ANALYSIS OF DATA

## Discussion, Results

Evaluations of the sampling and diluting errors were carried out for T1 and T2 phage titrations. Data from TABLES I and II reflected low coefficients of variance, indicating that there is little significant difference between the use of a 1.0 ml and 0.1 ml pipette for sampling and plating techniques.

Values for the probable error for each dilution series in each phage titration are given in TABLE III. There appears to be some differences between dilution series used. The probable error gradually increases as the number of dilution steps becomes greater. This has been reported by Luria and Darnell (1967), who state that the error is equal to the probable error of each step in the dilution series multiplied by the square root of the number of dilution steps.

TABLE I. Coefficient of Variance for T1 assay

| Plate number | Plaque count | Mean | s | cv |
|---|---|---|---|---|
| [a]1 | 50 | 47.8 | 6.4 | 13.4 |
| 2 | 43 | | | |
| 3 | 53 | | | |
| 4 | 43 | | | |
| 5 | 46 | | | |
| 6 | 41 | | | |
| 7 | 39 | | | |
| 8 | 55 | | | |
| 9 | 50 | | | |
| 10 | 58 | | | |
| [b]1 | 60 | 54.7 | 8.7 | 15.8 |
| 2 | 42 | | | |
| 3 | 56 | | | |
| 4 | 57 | | | |
| 5 | 43 | | | |
| 6 | 55 | | | |
| 7 | 56 | | | |
| 8 | 63 | | | |
| 9 | 70 | | | |
| 10 | 47 | | | |

[a] 1.0 ml pipette

[b] 0.1 ml pipette

TABLE II. Coefficient of Variance for T2 Assay

| Plate number | Plaque count | Mean | s | cv |
|---|---|---|---|---|
| [a]1 | 125 | 120.5 | 16.2 | 13.3 |
| 2 | 117 | | | |
| 3 | 129 | | | |
| 4 | 119 | | | |
| 5 | 130 | | | |
| 6 | 80 | | | |
| 7 | 122 | | | |
| 8 | 121 | | | |
| 9 | 138 | | | |
| 10 | 124 | | | |
| [b]1 | 122 | 127.5 | 8.8 | 6.9 |
| 2 | 139 | | | |
| 3 | 121 | | | |
| 4 | 120 | | | |
| 5 | 133 | | | |
| 6 | 118 | | | |
| 7 | 122 | | | |
| 8 | 128 | | | |
| 9 | 127 | | | |
| 10 | 145 | | | |

[a] 1.0 ml pipette

[b] 0.1 ml pipette

TABLE III. Probable Error for T1 and T2 Assay

| Phage | Dilution Series | PFU per ml | Mean | s | P.E. |
|---|---|---|---|---|---|
| T2 | 1:100 | $4.0 \times 10^6$ | 40.3 | 6.3 | 1.42 |
| | 1:10 | $4.7 \times 10^6$ | 46.8 | 11.9 | 2.88 |
| | [a]1:3 | $5.1 \times 10^6$ | 51.4 | 15.6 | 3.51 |
| T1 | 1:100 | $7.5 \times 10^6$ | 75.3 | 9.6 | 2.16 |
| | 1:10 | $8.1 \times 10^6$ | 80.7 | 10.1 | 2.27 |
| | [a]1:3 | $5.3 \times 10^6$ | 52.6 | 24.3 | 2.87 |

[a] Values for 1:3 dilution series included the dilution factor before calculations.

## UNRESTRICTED GROWTH

### Discussion, Results

When bacteria are inoculated into suitable medium and incubated under appropriate conditions, they begin to multiply and produce progeny, resulting in increased numbers in the population. Such growth is attributable to various physiological and cultural conditions previously described. One of these important variables, the nutritional environment, has been studied for its effect on bacterial growth. Comparisons of growth of Escherichia coli β were made in two types of media--a rich, nutrient medium, tryptose phosphate broth ($TPO_4$), and a synthetic or chemically-defined medium (M9).

Growth was monitored by optical density measurements as well as viable counts for a 24-hour period (TABLE I). Optical density curves shown in FIGURE 1 indicate a much higher cell concentration for the $TPO_4$-grown culture than for the M9-grown culture. It is also evident that growth occurred significantly earlier and was more rapid in $TPO_4$ than in M9. The viable cell data in FIGURE 2 (obtained from both media) further supports these observations.

Growth responses of the organism in both media were similar, i.e., the various growth phases were observable. The major difference was found in the extent of the initial lag period, which was about three times longer in M9 than in $TPO_4$. Generation times determined from the growth curves or calculated from the viable counts were compared for each culture -- 20 to 30 minutes in $TPO_4$ and 45 to 60 minutes in M9.

The fact that an organism was able to grow in a chemically-defined mixture indicated that it had an elaborate capacity for synthesizing these simple compounds into more complex compounds. The longer apparent lag period in M9 medium may be attributable to the fact that the organism must first produce the enzymes needed for optimal operation of the cell machinery to prepare further growth nutrients. In an enriched medium, most of the nutrients are available for cells to initiate earlier growth.

Bacterial cultures exhibit continuous variations in growth, being sensitive to fluctuations in the chemical and physical environment.

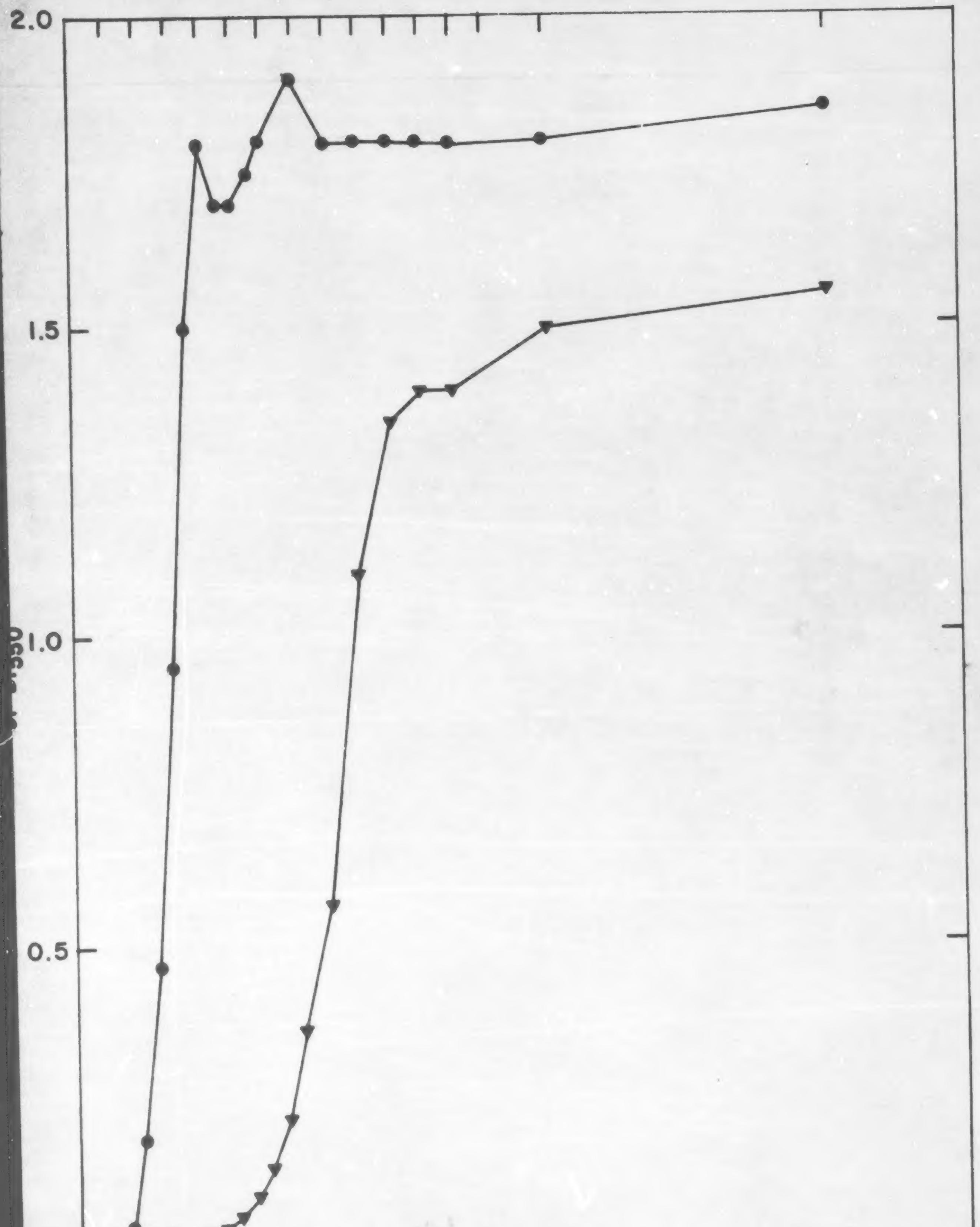
Optical density for _E_. _coli_ β cultures in TPO4 and M9 media.

Figure 1.

The bacterial culture was prepared by a 1:200 dilution of an overnight culture of _E_. _coli_ β into fresh, pre-warmed media (TPO$_4$ or M9). Incubation was made at 37 C. Samples of 4.5 mls of the bacterial culture were taken at indicated time intervals and determined for optical density on a spectrophotometer at a wavelength of 550 mμ.

● TPO4

▼ M9


2.0
1.5
1.0
0.5
2
4
6
8
10
12
15
24
TIME (HRS)

Colony-forming cells for E. coli β cultures in TPO4 and M9 media.

Figure 2.

Samples of 0.5 ml were taken at the different times, diluted appropriately, and assayed for viable counts by the spread plate method, using 5 replicate plates per dilution.

● TPO4

▼ M9

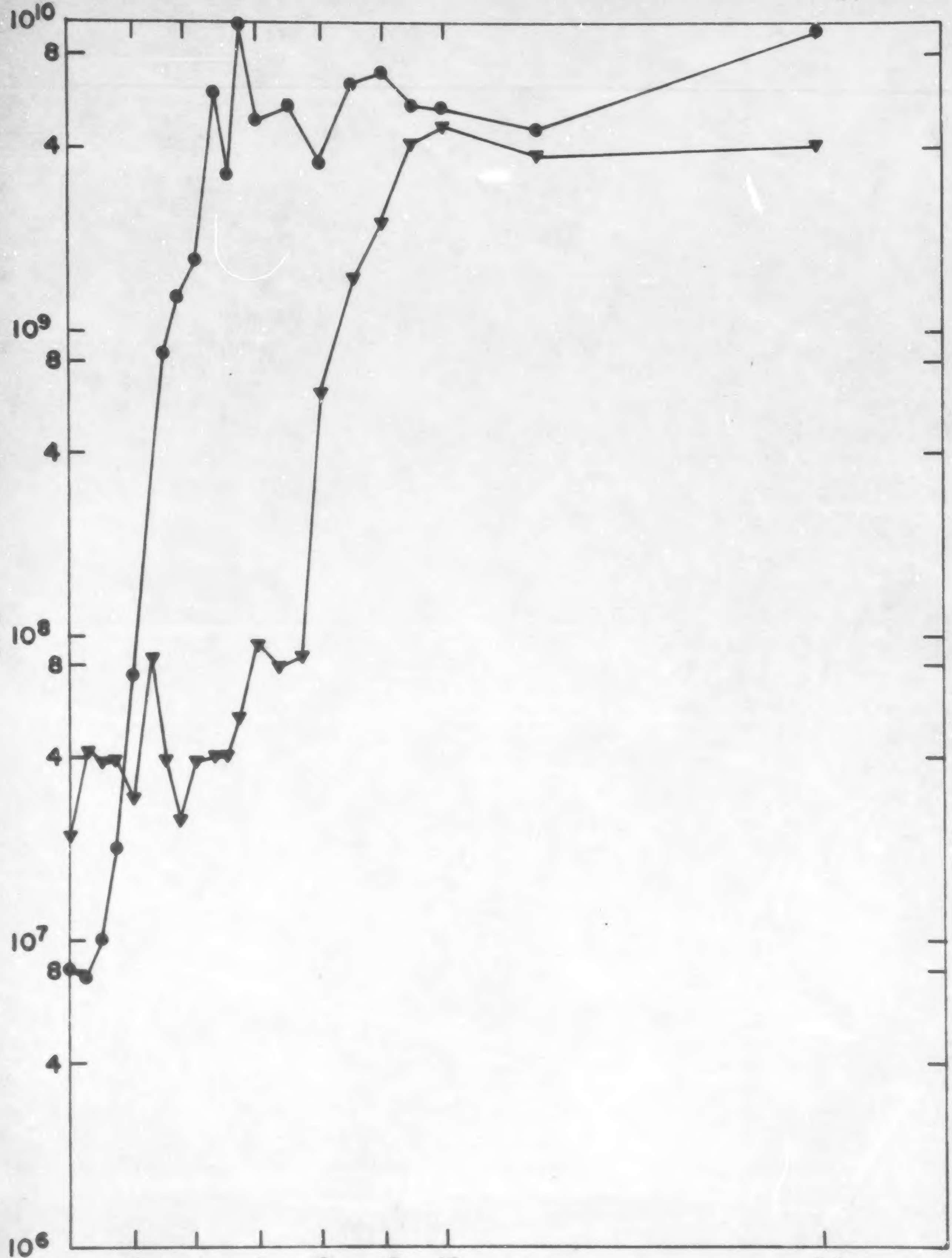

10^10
8
4
10^9
8
4
10^8
8
4
10^7
8
4
10^6
CFU
2
4
6
8
10
12
24
TIME (HRS)

TABLE I. Growth Curve of *Escherichia coli* β

| Time (hours) | Optical Density (550 mμ) |  | Bacteria per ml |  |
|---|---|---|---|---|
|  | TPO$_4$ | M9 | TPO$_4$ | M9 |
| 0 | 0.018 | 0.01 | $8.0 \times 10^6$ | $2.2 \times 10^7$ |
| 0.5 | 0.018 | 0.01 | $7.5 \times 10^6$ | $4.2 \times 10^7$ |
| 1.0 | 0.022 | 0.029 | $1.0 \times 10^7$ | $3.8 \times 10^7$ |
| 1.5 | 0.051 | 0.026 | $2.0 \times 10^7$ | $3.9 \times 10^7$ |
| 2.0 | 0.191 | 0.038 | $7.5 \times 10^7$ | $2.9 \times 10^7$ |
| 2.5 | 0.47 | 0.03 | $1.5 \times 10^8$ | $8.5 \times 10^7$ |
| 3.0 | 0.95 | 0.033 | $8.5 \times 10^8$ | $4.0 \times 10^7$ |
| 3.5 | 1.5 | 0.03 | $1.3 \times 10^9$ | $2.5 \times 10^7$ |
| 4.0 | 1.8 | 0.041 | $1.7 \times 10^9$ | $3.9 \times 10^7$ |
| 4.5 | 1.7 | 0.05 | $6.0 \times 10^9$ | $4.0 \times 10^7$ |
| 5.0 | 1.7 | 0.068 | $3.3 \times 10^9$ | $4.1 \times 10^7$ |
| 5.5 | 1.75 | 0.099 | $1.0 \times 10^{10}$ | $5.5 \times 10^7$ |
| 6.0 | 1.8 | 0.14 | $4.8 \times 10^9$ | $9.5 \times 10^7$ |
| 6.5 | - | 0.219 | - | $8.0 \times 10^7$ |
| 7.0 | 1.9 | 0.361 | $5.5 \times 10^9$ | $8.5 \times 10^7$ |
| 8.0 | 1.8 | 0.561 | $3.6 \times 10^9$ | $6.5 \times 10^8$ |
| 9.0 | 1.8 | 1.1 | $6.5 \times 10^9$ | $1.5 \times 10^9$ |
| 10.0 | 1.8 | 1.35 | $7.0 \times 10^9$ | $2.3 \times 10^9$ |
| 11.0 | 1.8 | 1.4 | $5.5 \times 10^9$ | $4.4 \times 10^9$ |
| 12.0 | 1.8 | 1.4 | $5.5 \times 10^9$ | $4.8 \times 10^9$ |
| 15.0 | 1.8 | 1.5 | $4.6 \times 10^9$ | $3.8 \times 10^9$ |
| 24.0 | 1.85 | 1.55 | $9.5 \times 10^9$ | $4.1 \times 10^9$ |

# ONE-STEP GROWTH OF A BACTERIOPHAGE

## Discussion, Results

The one-step growth experiment has been described to provide a useful scheme to monitor the various stages of phage development after infection. Growth was followed by infectivity assays during the transition period to the production of progeny phage.

FIGURE 1 shows the one-step growth profile for T2 phage. Infected centers were assayed at indicated time intervals (TABLE I). Unadsorbed phage particles were neutralized by phage-specific antisera. Definitive growth phases, characteristic of the normal viral growth, are observable from the plotted data. The duration of the latent period is approximately 20 minutes before the onset of virus release. The final burst size is completed by 30 minutes with subsequent leveling of phage numbers. Should cell concentrations be larger than the virus inoculum, susceptible cells are still available for infection, thereby creating the stepwise nature of viral growth.

Comparisons of growth curve profiles were conducted for normal growth with that obtained from premature lysis of infected cells (TABLE II). This would enable some characterization of the latent period. FIGURE 2 indicates results obtained for T4 when chloroformed phage-infected samples were assayed at different times. As expected, values obtained from the premature lysis of infected cells were higher and mature virions were seen earlier, since most virions have been assembled and awaiting for release. Samples must be shaken vigorously, however, to lyse cells to obtain maximal recovery.

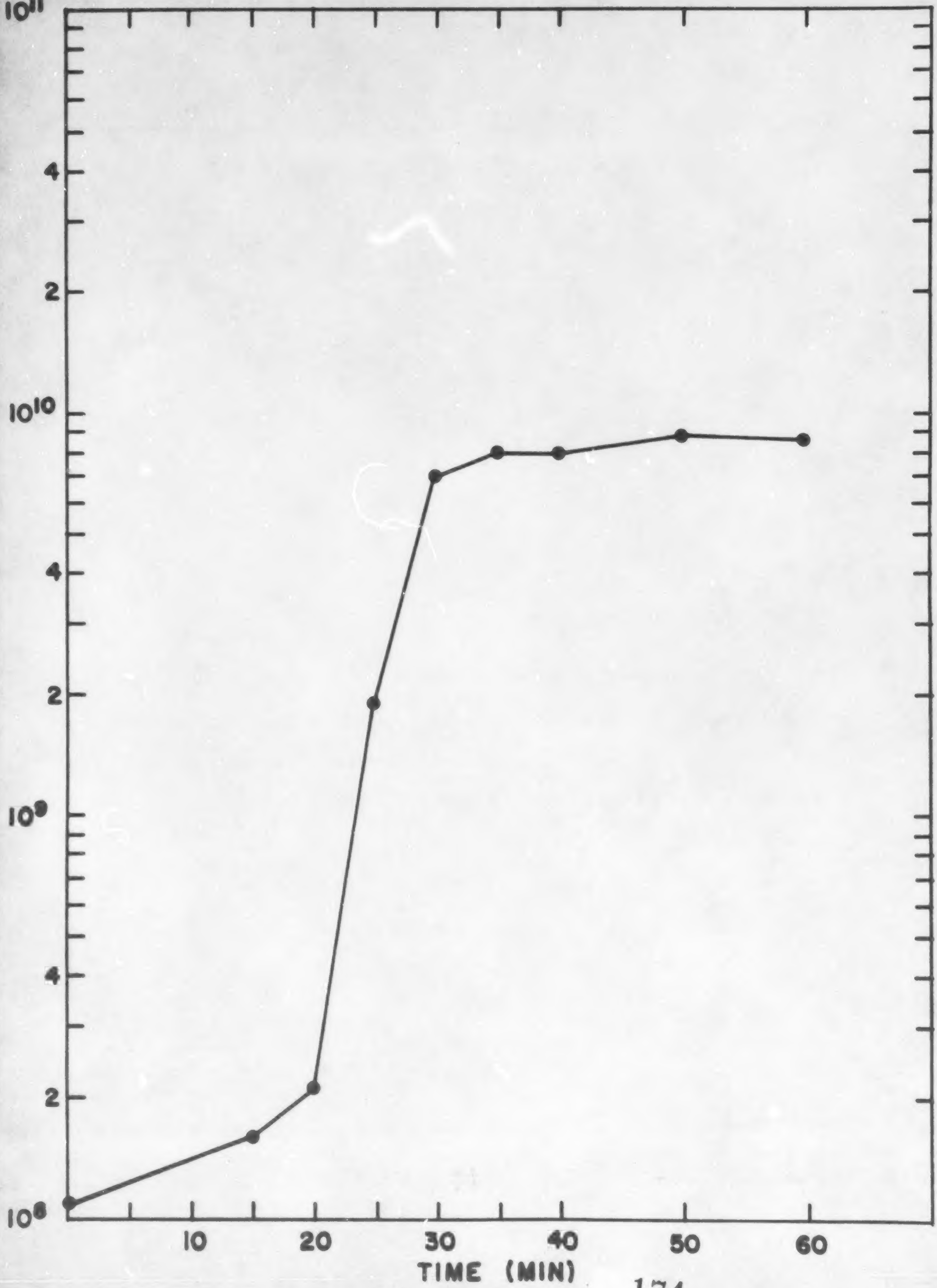
It is interesting to note the similarities and differences between the one-step growth of a virus and the growth of a bacterium. Both have a comparable initial period (latent, lag), a prominent growth period (rise, exponential), and finally, a stationary phase. Viral growth will be influenced by factors that affect bacterial growth e.g., environmental and nutritional conditions, temperature, pH, and other factors. There are unique differences, however. One is reflected in the duration of the growth cycle, minutes as opposed to hours. More distinctly, bacterial cell division proceeds by geometric progression, i.e., doubling, whereas phage reproduction as manifested in free virions results from a burst of several hundred or thousand progeny phages from each infected cell.

Such experiments have facilitated the growth studies of different viruses, virus-host interactions, as well as investigating various genetic relationships.

One step growth of bacteriophage T2.

Figure 1.

At time zero, T2 phage ($5 \times 10^7$ PFU/ml) was added to a logarithmic broth culture of E. coli β (2.5 hr. $6 \times 10^8$ bacteria/ml) at 37°C. A five minute adsorption period was allowed before incubation in antisera for five minutes. The mixture was further diluted 1:500 to prevent additional attachment. Samples were appropriately diluted and assayed for infectious centers at the indicated time intervals (●).


10^11
4
2
10^10
4
2
10^9
4
2
10^8
10
20
30
40
50
60
TIME (MIN)

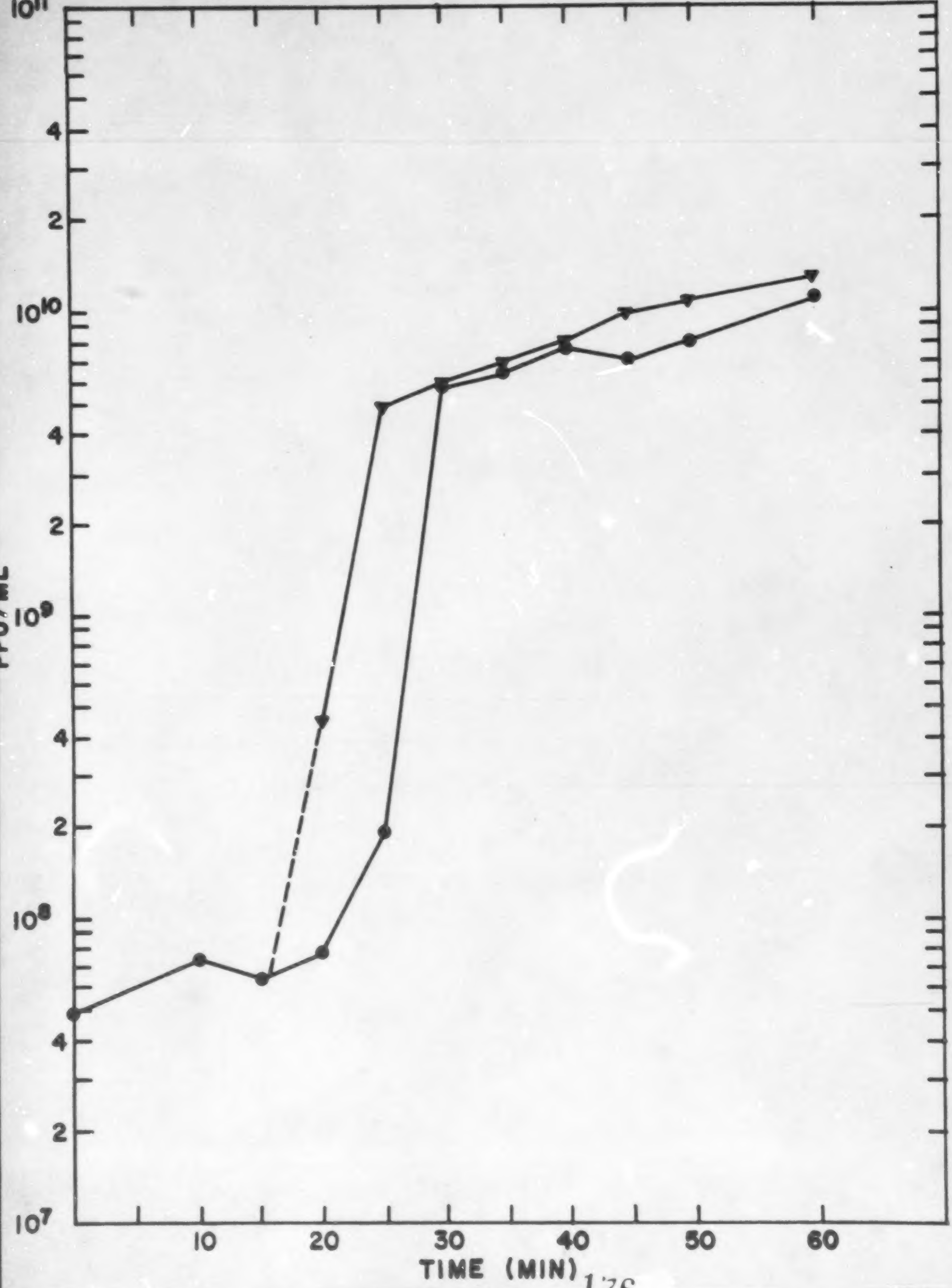
One step growth of bacteriophage T4.

Figure 2.

The same experimental procedures as in FIGURE 1. Additional second samples were taken and vigorously shaken with chloroform-broth mixtures. The separated aqueous layer was diluted and also assayed for infected centers at the same time intervals as the normal phage growth samples.

● Procedure

▼ Time Intervals



$10^{11}$
4
2
$10^{10}$
4
2
$10^{9}$
4
2
$10^{8}$
4
2
$10^{7}$
10
20
30
40
50
60
TIME (MIN)

TABLE I. One-Step Growth of T2 phage at 37°C

| Time (Min.) | First Growth Tube* (PFU per ml) | Second Growth Tube** (PFU per ml) |
|---|---|---|
| 15 | $1.6 \times 10^8$ | -- |
| 20 | $2.1 \times 10^8$ | -- |
| 25 | TNTC | $1.9 \times 10^9$ |
| 30 | TNTC | $7.0 \times 10^9$ |
| 35 | TNTC | $8.0 \times 10^9$ |
| 40 | TNTC | $8.0 \times 10^9$ |
| 50 | TNTC | $8.8 \times 10^9$ |
| 60 | TNTC | $8.6 \times 10^9$ |

Data plotted as one-step growth curve (FIGURE 1).

*Appropriate dilution factor, $5 \times 10^5$

**Appropriate dilution factor, $5 \times 10^7$

TABLE II. One-Step Growth of T4 phage at 37°C

| Time (Min.) | FGT PFU/ml | SGT PFU/ml | FGT ($CHCl_3$) PFU/ml |
|---|---|---|---|
| 10 | $7.4 \times 10^7$ | -- | 0 |
| 15 | $6.4 \times 10^7$ | -- | 0 |
| 20 | $7.8 \times 10^7$ | -- | $4.5 \times 10^8$ |
| 25 | TNTC | $1.9 \times 10^9$ | $4.9 \times 10^9$ |
| 30 | TNTC | $5.7 \times 10^9$ | $5.8 \times 10^9$ |
| 35 | TNTC | $6.5 \times 10^9$ | $7.0 \times 10^9$ |
| 40 | TNTC | $7.7 \times 10^9$ | $7.7 \times 10^9$ |
| 45 | TNTC | $7.2 \times 10^9$ | $1.0 \times 10^{10}$ |
| 50 | TNTC | $8.1 \times 10^9$ | $1.1 \times 10^{10}$ |
| 60 | TNTC | $1.1 \times 10^{10}$ | $1.3 \times 10^{10}$ |

Data plotted as one-step growth curve (FIGURE 2).

Appropriate dilution factor, FGT, $5 \times 10^5$

SGT, $5 \times 10^7$

FGT ($CHCl_3$), $5 \times 10^6$

# ADSORPTION OF VIRUS TO A BACTERIUM

## Discussion, Results

The adsorption rate of a virus particle to a bacterium has been observed to be a function of virus and cell concentration and reaction time. These observations were investigated by conducting attachment experiments, monitoring the infectivity of phage-bacterium mixtures with varying cell and virus concentrations at different time intervals.

Figures 1-3 indicate the profiles obtained when decreasing cell densities were infected by constant virus concentrations. Infected center and free phage levels were determined and compared to those obtained at the control cell density -- a 2.5 hour bacterial culture containing 3-5 × $10^8$ cells per ml. Reaction times and maximal adsorption rates appear similar for each titration (Figures 1 and 2). The majority of the phage particles (90-99%) have attached within 5 minutes after mixing. Observe that there is a corresponding decrease in the number of free phages. Similar results were obtained when increasing cell concentrations were employed (Figure 4). It must be considered that further dilution of cell density, however, will pose a limiting end-point in which significant differences between time and adsorption rate is apparent (Figure 3). Any additional cell dilutions below 1:300 will probably decrease the number of infected centers observed, thereby altering the curve profiles significantly.

Varying the virus concentrations with a constant cell density indicates little difference in adsorption time rate except for the expected 100-fold difference in concentrations (FIGURE 6).

Any difference in adsorption rates can be examined by determination of the rate constant from the plotted data. TABLE I reflects the K-values for the various cell concentrations employed, the rate being proportional to the cell density. TABLE II indicates that K-values were almost identical for the two varying virus concentrations.

Studies were also initiated to determine whether physiological changes in the cell had any effects on adsorption rates. It has been shown that cells undergo physical changes in size during the first few hours of growth, especially along the longitudinal axis, with the average length being several times that of the inoculated cells. These eventually return to the original size after entering the logarithmic phage of growth (ref. 8 under "Unrestricted Growth").

A young culture was prepared by dilution from an overnight culture and sampled at various stages of growth. The results are shown in Figure 5 and K-values in TABLE III. Since cell and virus concentrations used in each titration were approximately the same, there is still observed some differences in the number of infected cells with corresponding differences in free phages. If the increased size changes is taken into consideration, as those sampled at 1.6 and 2.5 hours, this factor may contribute to increase the collision frequency of phage particles to the bacterial cells. By 12 hours, however, the cells have returned to the original size as the inoculum and would not demonstrate the size change. Other studies may be conducted to determine the major size groups contained in the different cultures, e.g., by use of an electronic Coulter counter, and then further evaluated.

Phage adsorption occurs as a very rapid phenomenon, taking place within minutes after contact with susceptible cells. It should be remembered that a cycle of phage growth will commence shortly with liberation of progeny phage. Measurements must be made within the initial period of infection. Otherwise, newly released phage particles will interfere with determinations (FIGURE 5), especially shown as an increase in free phage with a corresponding increase in the number of infected centers.

Figures 1-6.

Young E. coli β cultures (2.5 hrs.) were prepared in broth. Increasing or decreasing cell densities were achieved by centrifugation or dilution of cultures. Cell pellets obtained were resuspended in equal volumes of media.

At time zero, phage was added to the designated broth cultures at 37°C. Samples were assayed at various time intervals. Chloroformed-samples appropriately diluted and assayed for free phages, and antisera-treated samples diluted for assay of infected centers.

Figure 1. Adsorption of T4 to decreasing concentration of bacteria.

●, ○ $5.6 \times 10^8$ bacteria/ml

■, □ $1.2 \times 10^8$ bacteria/ml

▼, ▽ $5.0 \times 10^7$ bacteria/ml

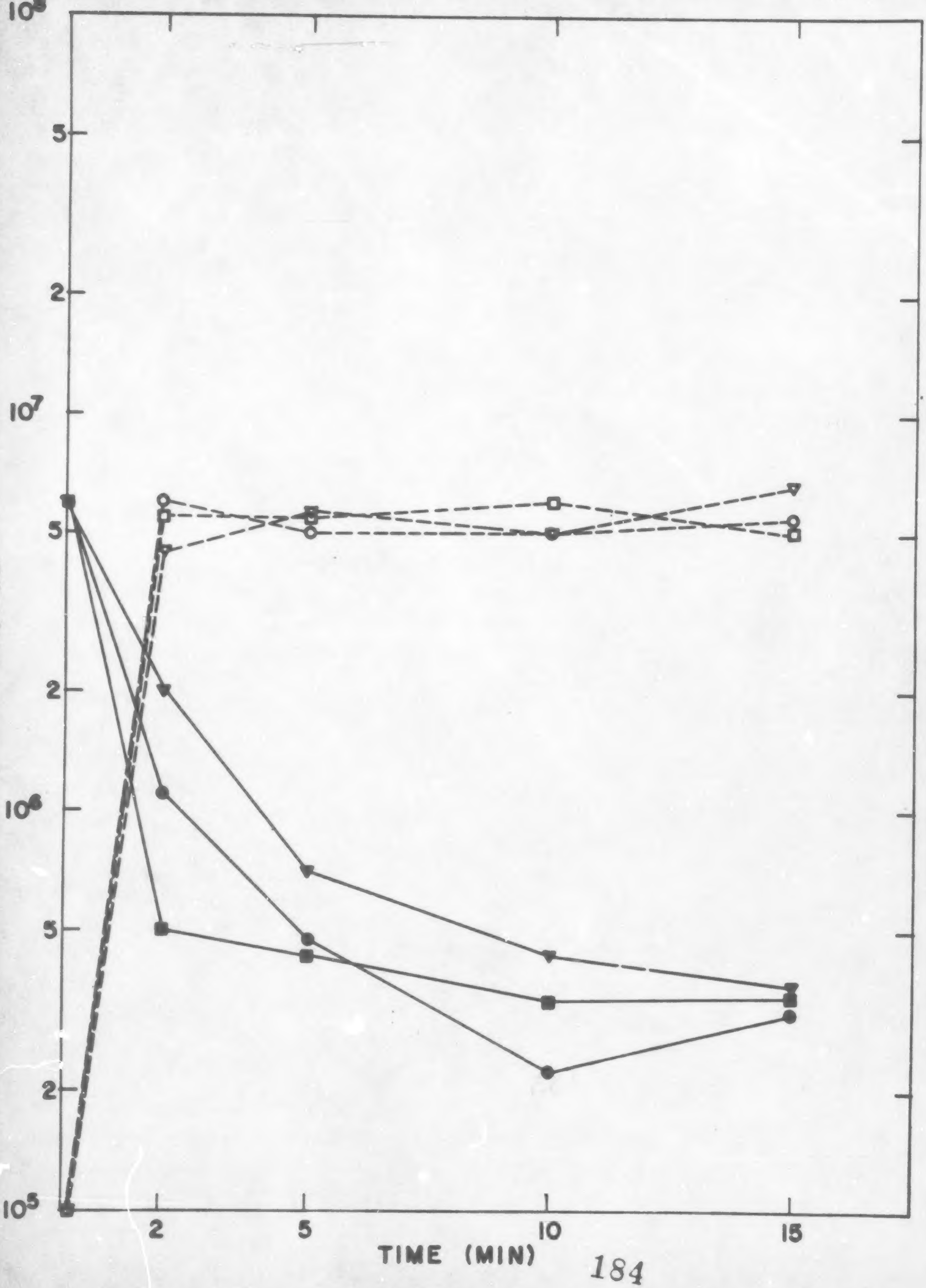

$10^8$
5
2
$10^7$
5
2
$10^6$
5
2
$10^5$
2
5
10
15
TIME (MIN)

Figure 2. Adsorption of T4 to decreasing concentrations of bacteria.

●, ○ $3.5 \times 10^8$ bacteria/ml

■, □ $3.5 \times 10^7$ bacteria/ml

▼, ▽ $9.0 \times 10^6$ bacteria/ml

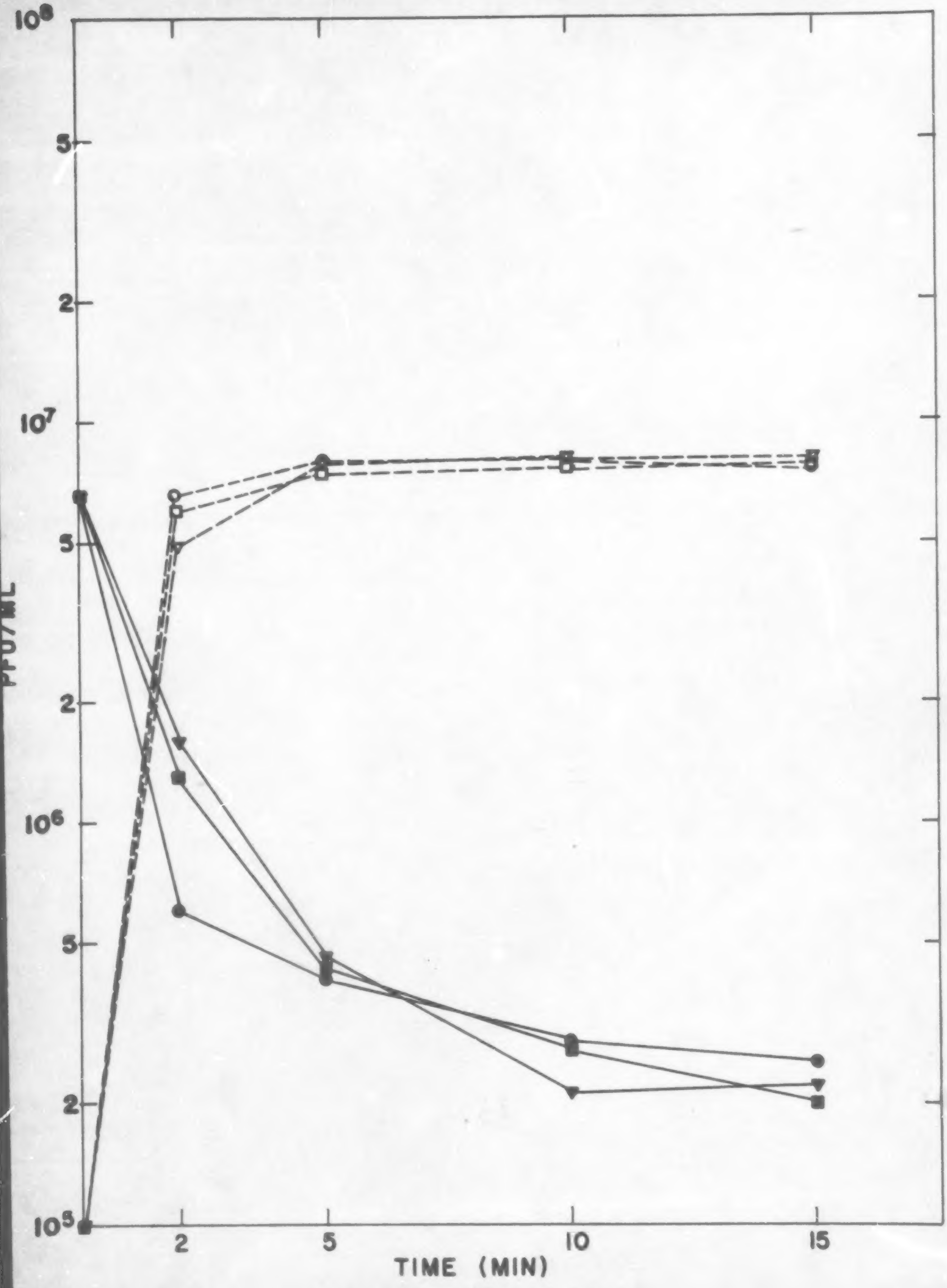

10^8
5
2
10^7
5
2
10^6
5
2
10^5
PFU/ML
2
5
10
15
TIME (MIN)

Figure 3. Adsorption of T4 to decreasing concentrations of bacteria.

●, ○ $3.6 \times 10^8$ bacteria/ml

■, □ $1.6 \times 10^6$ bacteria/ml

▼, ▽ $1.3 \times 10^6$ bacteria/ml

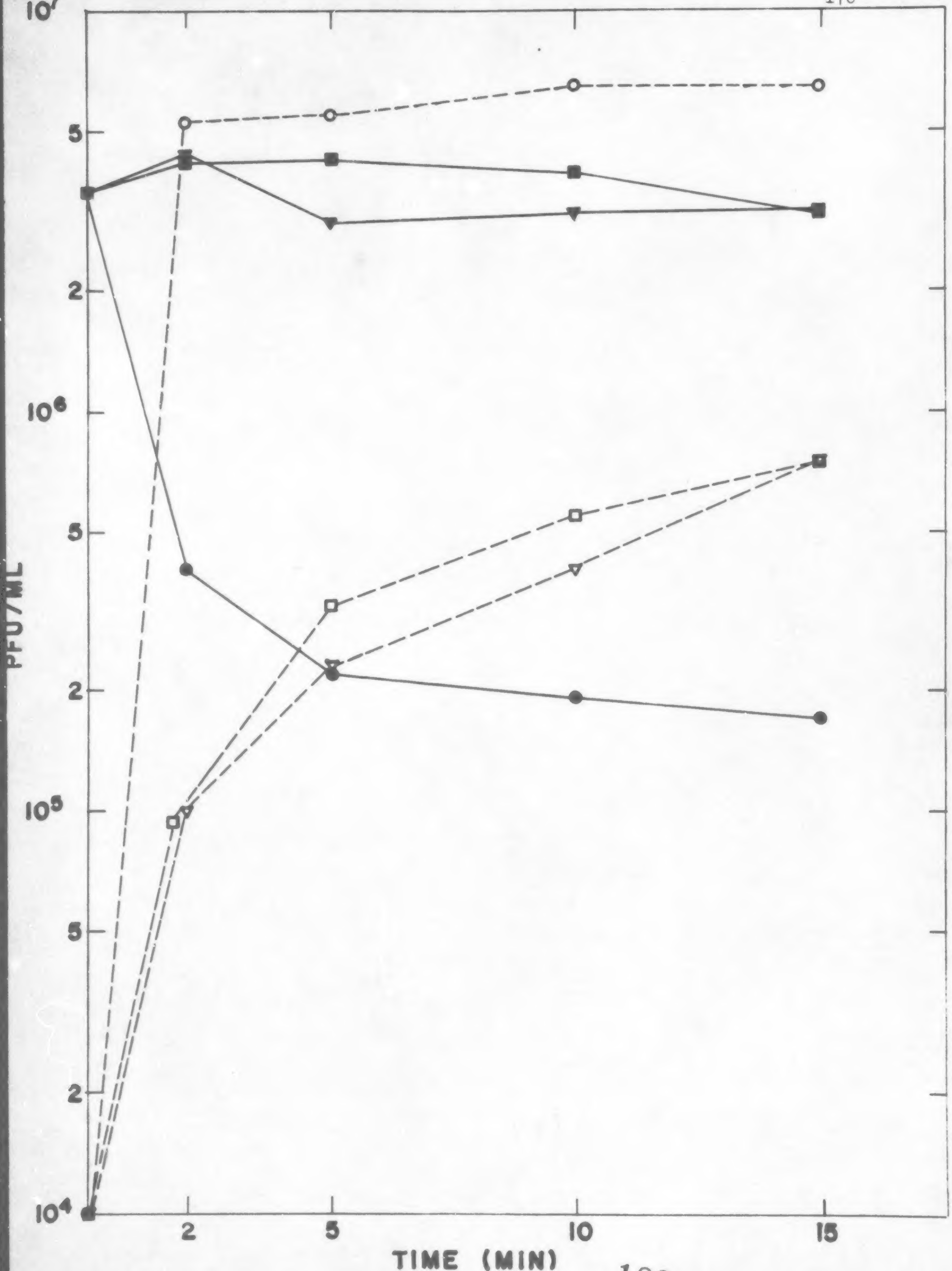

10^7
5
2
10^6
5
2
10^5
5
2
10^4
2
5
10
15
TIME (MIN)
PFU/ML

Figure 4. Adsorption of T4 to increasing concentrations of bacteria.

●, ○ $4.1 \times 10^8$ bacteria/ml

■, □ $8.5 \times 10^8$ bacteria/ml

▼, ▽ $2.6 \times 10^9$ bacteria/ml

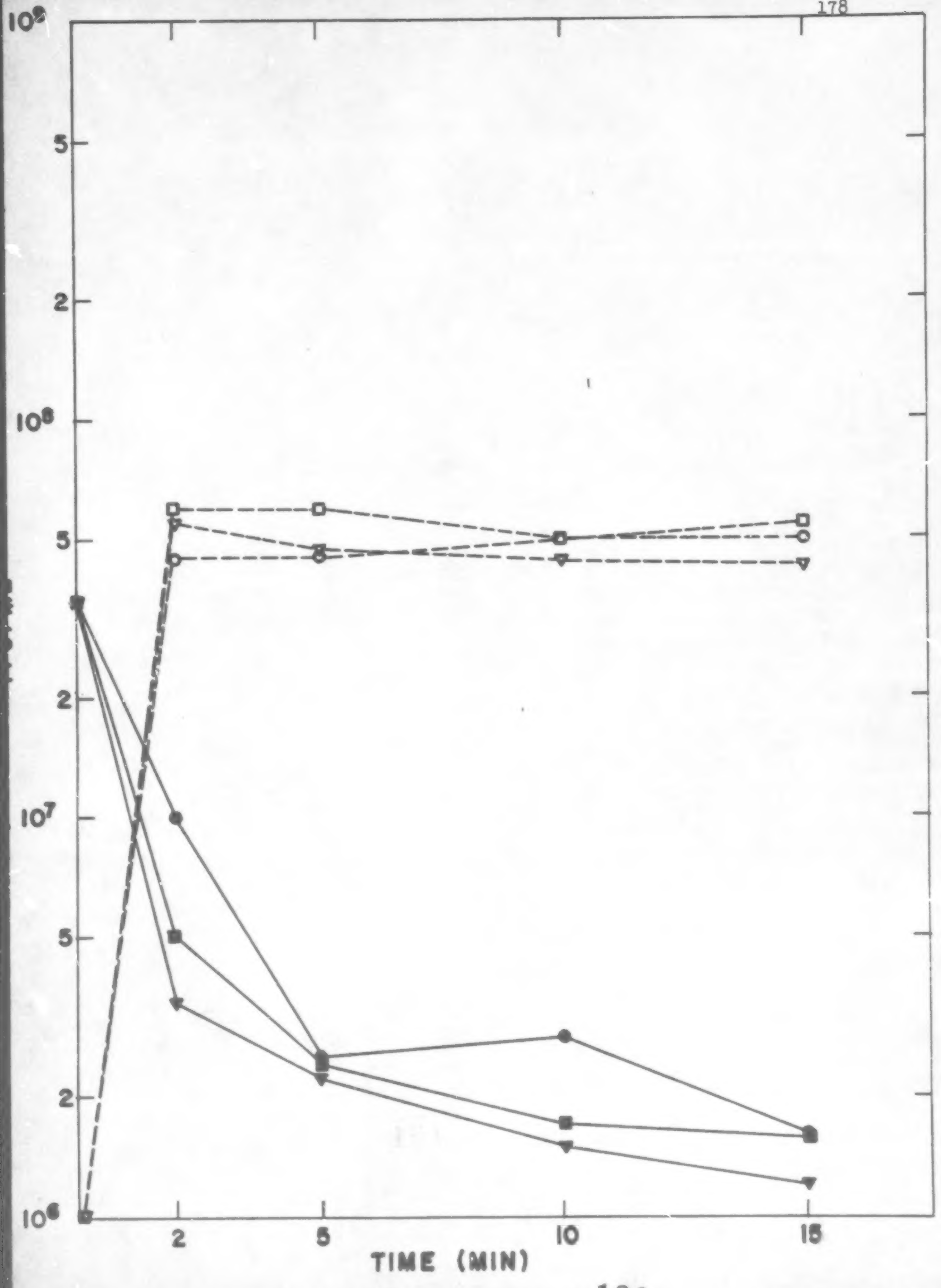

10⁹
5
2
10⁸
5
2
10⁷
5
2
10⁶
2
5
10
15
TIME (MIN)

Figure 5. Adsorption of T4 to varying ages of bacteria.

●, ○ 1.6 hours

■, □ 2.5 hours

▼, ▽ 12 hours

Note: Figures 1-5, open symbols indicate infected centers (IC) and closed symbols indicate infectious free phage.

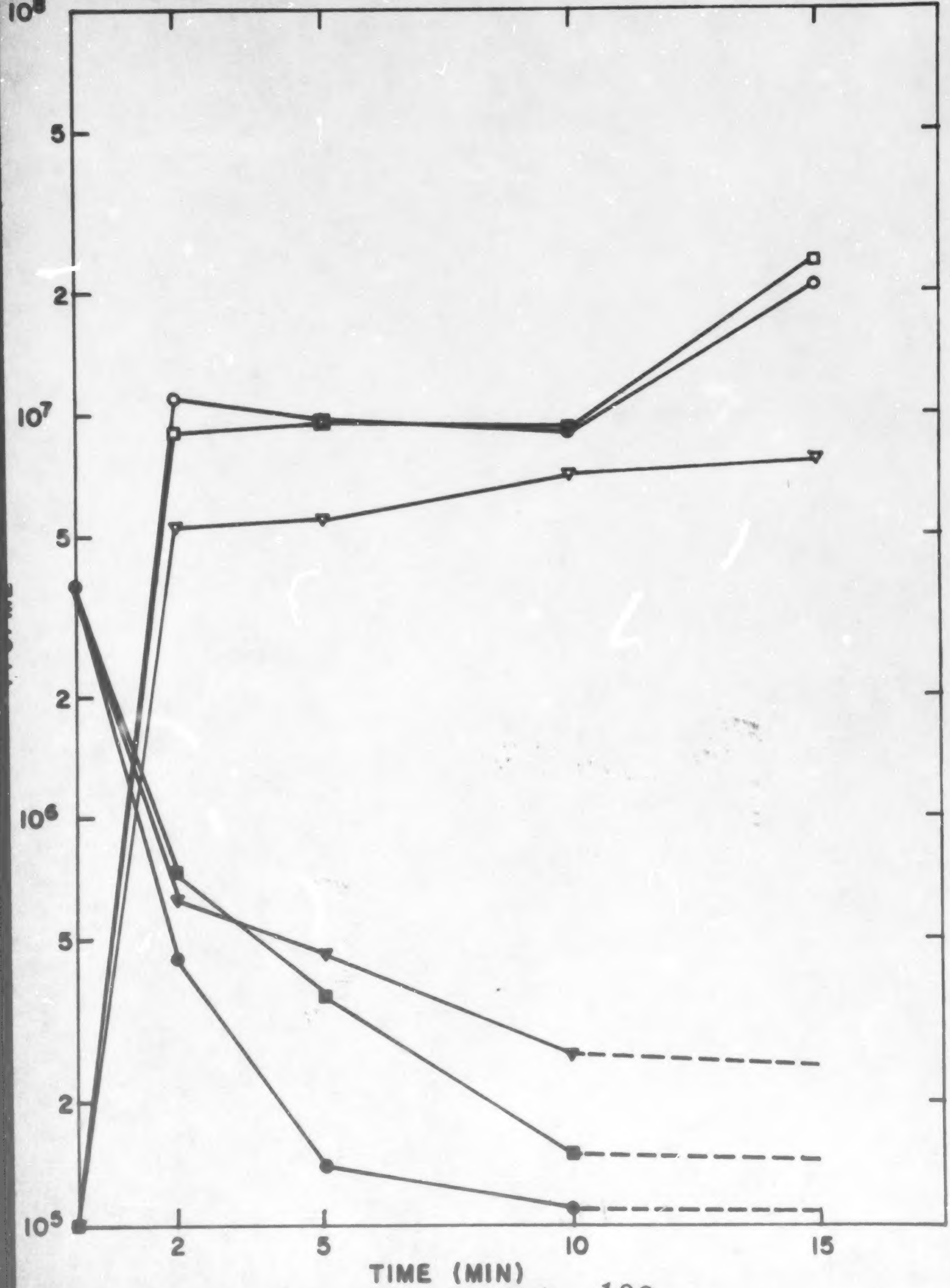

$10^8$
5
2
$10^7$
5
2
$10^6$
5
2
$10^5$
2
5
10
15
TIME (MIN)

Figure 6. Adsorption of varying concentrations of T4 to bacteria.

●——● $10^5$, free phage

●---● $10^5$, IC

▼——▼ $10^7$, free phage

▼---▼ $10^7$, IC

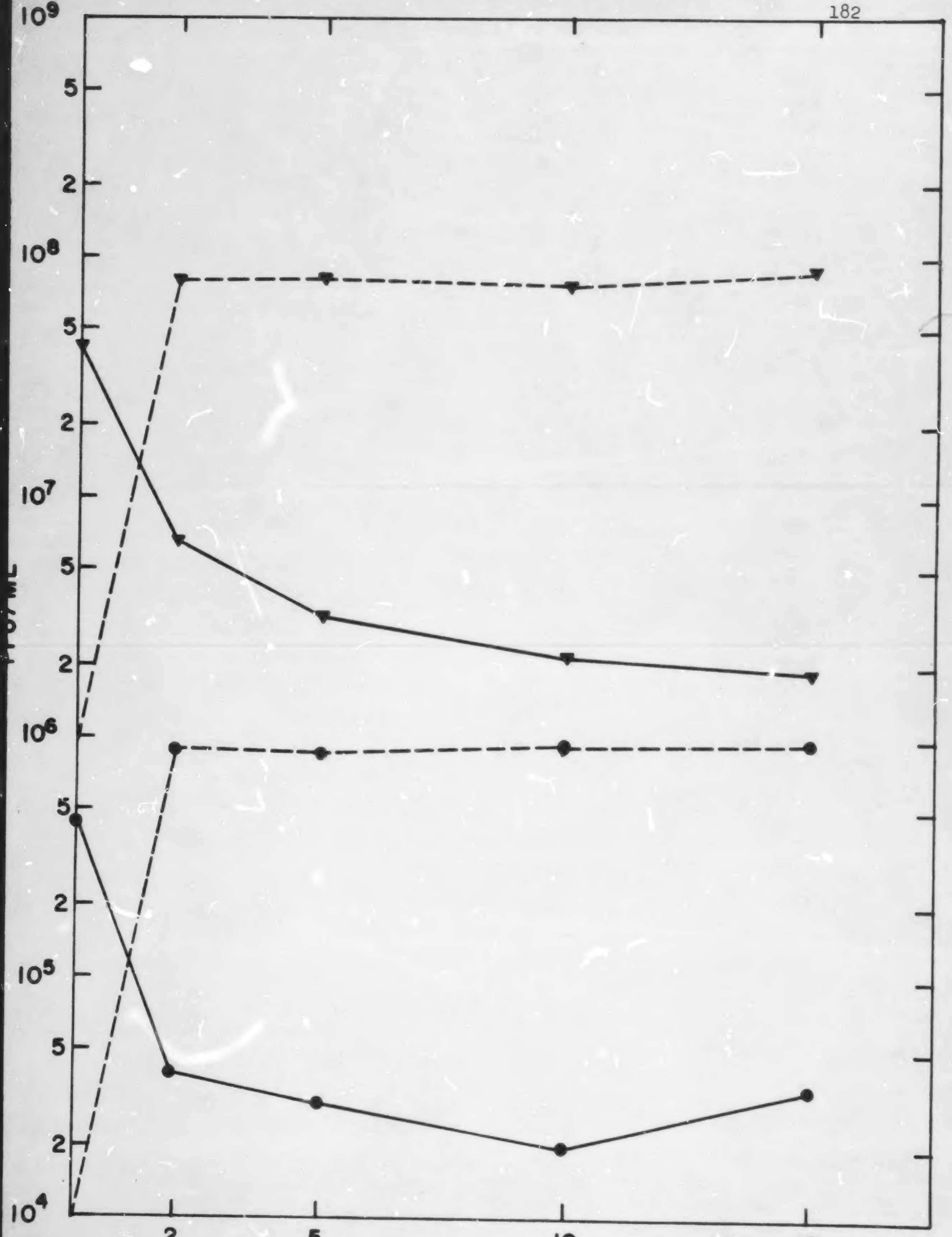

10⁹
5
2
10⁸
5
2
10⁷
5
2
10⁶
5
2
10⁵
5
2
10⁴
2
5
10
15
TIME (MIN)

TABLE I. Varying Cell Concentrations

| Cell concentration bacteria/ml | Virus concentration PFU/ml | K-value |
|---|---|---|
| $5.6 \times 10^{8}$ | $6.0 \times 10^{6}$ | $0.9 \times 10^{-9}$ |
| $1.2 \times 10^{8}$ | $6.0 \times 10^{6}$ | $4.3 \times 10^{-9}$ |
| $5.0 \times 10^{7}$ | $6.0 \times 10^{6}$ | $8.6 \times 10^{-9}$ |
| $3.5 \times 10^{8}$ | $6.5 \times 10^{6}$ | $1.5 \times 10^{-9}$ |
| $3.5 \times 10^{7}$ | $6.5 \times 10^{6}$ | $1.5 \times 10^{-10}$ |
| $9.0 \times 10^{6}$ | $6.5 \times 10^{6}$ | $6.0 \times 10^{-10}$ |
| $3.6 \times 10^{8}$ | $3.5 \times 10^{6}$ | $1.5 \times 10^{-9}$ |
| $1.6 \times 10^{6}$ | $3.5 \times 10^{6}$ | $^{*}3.4 \times 10^{-10}$ |
| $1.3 \times 10^{6}$ | $3.5 \times 10^{6}$ | $2.6 \times 10^{-10}$ |
| $4.1 \times 10^{8}$ | $3.5 \times 10^{7}$ | $1.3 \times 10^{-9}$ |
| $8.5 \times 10^{8}$ | $3.5 \times 10^{7}$ | $0.6 \times 10^{-9}$ |
| $2.6 \times 10^{9}$ | $3.5 \times 10^{7}$ | $0.2 \times 10^{-9}$ |

K-values determined at t = 5 min.
*Determined at t = 15 min.

TABLE II. Varying Virus Concentrations.

| Virus concentration PFU/ml | Cell concentration bacteria/ml | K-value |
|---|---|---|
| $4.4 \times 10^5$ | $4.9 \times 10^8$ | $1.1 \times 10^{-9}$ |
| $4.2 \times 10^7$ | $4.9 \times 10^8$ | $1.0 \times 10^{-9}$ |

K-value determined at t = 5 min.

Table III. Varying Ages of Cell Cultures

| Age of Culture | Cell Density Bacteria/ml | Virus Concentration PFU/ml | K-value |
|---|---|---|---|
| 1.6 hrs. | $2.5 \times 10^8$ | $3.8 \times 10^6$ | $2.2 \times 10^{-9}$ |
| 2.5 hrs. | $2.7 \times 10^8$ | $3.8 \times 10^6$ | $1.7 \times 10^{-9}$ |
| 12 hrs. | $2.6 \times 10^8$ | $3.8 \times 10^6$ | $1.6 \times 10^{-9}$ |

K-values determined at $t = 5$ min.

TABLE IV. Adsorption of Virus at Decreasing Cell Densities -- Infected Center and Unadsorbed Phage Assays

| Cell Concentration Bacteria per ml | Dilution | Time (Min.) | PFU per ml Infected Centers | PFU per ml Unadsorbed Phage |
|---|---|---|---|---|
| $5.6 \times 10^8$ | 0 | 2 | $6.0 \times 10^6$ | $1.1 \times 10^6$ |
| | | 5 | $5.0 \times 10^6$ | $4.7 \times 10^5$ |
| | | 10 | $5.0 \times 10^6$ | $2.2 \times 10^5$ |
| | | 15 | $5.5 \times 10^5$ | $3.1 \times 10^5$ |
| $1.2 \times 10^8$ | 1:3 | 2 | $5.5 \times 10^6$ | $5.0 \times 10^5$ |
| | | 5 | $5.5 \times 10^6$ | $4.3 \times 10^5$ |
| | | 10 | $6.0 \times 10^6$ | $3.3 \times 10^5$ |
| | | 15 | $5.0 \times 10^6$ | $3.4 \times 10^5$ |
| $5.0 \times 10^7$ | 1:10 | 2 | $4.5 \times 10^6$ | $2.0 \times 10^6$ |
| | | 5 | $5.7 \times 10^6$ | $7.0 \times 10^5$ |
| | | 10 | $5.0 \times 10^6$ | $4.3 \times 10^5$ |
| | | 15 | $6.6 \times 10^6$ | $3.6 \times 10^5$ |

Data plotted as adsorption curve (FIGURE 1).

TABLE IVa. Adsorption of Virus at Decreasing Cell Densities -- Infected Center and Uradsorbed Phage Assays

| Cell Concentration Bacteria per ml | Dilution | Time (Min.) | PFU per ml | |
|---|---|---|---|---|
| | | | Infected Centers | Unadsorbed Phage |
| $3.5 \times 10^8$ | 0 | 2 | $6.5 \times 10^6$ | $6.0 \times 10^5$ |
| | | 5 | $8.0 \times 10^6$ | $4.0 \times 10^5$ |
| | | 10 | $8.0 \times 10^6$ | $2.8 \times 10^5$ |
| | | 15 | $7.5 \times 10^6$ | $2.5 \times 10^5$ |
| $3.5 \times 10^7$ | 1:10 | 2 | $4.9 \times 10^6$ | $1.0 \times 10^6$ |
| | | 5 | $8.0 \times 10^6$ | $4.6 \times 10^5$ |
| | | 10 | $8.0 \times 10^6$ | $2.1 \times 10^5$ |
| | | 15 | $8.0 \times 10^6$ | $2.2 \times 10^5$ |
| $9.0 \times 10^6$ | 1:50 | 2 | $6.0 \times 10^6$ | $1.3 \times 10^6$ |
| | | 5 | $7.5 \times 10^6$ | $4.4 \times 10^5$ |
| | | 10 | $8.0 \times 10^6$ | $2.7 \times 10^5$ |
| | | 15 | $8.5 \times 10^6$ | $2.0 \times 10^5$ |

Data plotted as adsorption curve (FIGURE 2).

TABLE IVb. Adsorption of Virus at Decreasing Cell Densities -- Infected Center and Unadsorbed Phage Assays

| Cell Concentration Bacteria per ml | Dilution | Time (min.) | PFU per ml Infected Centers | PFU per ml Unadsorbed Phage |
|---|---|---|---|---|
| $3.6 \times 10^8$ | 0 | 2 | $5.3 \times 10^6$ | $4.0 \times 10^5$ |
| | | 5 | $5.5 \times 10^6$ | $2.2 \times 10^5$ |
| | | 10 | $6.5 \times 10^6$ | $1.9 \times 10^5$ |
| | | 15 | $6.5 \times 10^6$ | $1.7 \times 10^5$ |
| $1.6 \times 10^6$ | 1:225 | 2 | $9.0 \times 10^4$ | $4.2 \times 10^6$ |
| | | 5 | $3.3 \times 10^5$ | $4.3 \times 10^6$ |
| | | 10 | $5.5 \times 10^5$ | $4.0 \times 10^6$ |
| | | 15 | $7.5 \times 10^5$ | $3.3 \times 10^6$ |
| $1.3 \times 10^6$ | 1:300 | 2 | $1.0 \times 10^5$ | $4.4 \times 10^6$ |
| | | 5 | $2.3 \times 10^5$ | $3.0 \times 10^6$ |
| | | 10 | $4.0 \times 10^5$ | $3.4 \times 10^6$ |
| | | 15 | $7.5 \times 10^5$ | $3.4 \times 10^6$ |

Data plotted as adsorption curve (FIGURE 3).

TABLE V. Adsorption of Virus at Increasing Cell Densities -- Infected Center and Unadsorbed Phages Assays

| Cell Concentration Bacteria per ml | Dilution | Time (Min.) | PFU per ml Infected Centers | PFU per ml Unadsorbed Phage |
|---|---|---|---|---|
| $4.1 \times 10^8$ | 0 | 2 | $4.5 \times 10^7$ | $1.0 \times 10^7$ |
| | | 5 | $4.5 \times 10^7$ | $2.5 \times 10^6$ |
| | | 10 | $5.0 \times 10^7$ | $2.8 \times 10^6$ |
| | | 15 | $5.5 \times 10^7$ | $1.6 \times 10^6$ |
| $2.5 \times 10^8$ | 2:1 | 2 | $6.0 \times 10^7$ | $5.0 \times 10^6$ |
| | | 5 | $6.0 \times 10^7$ | $2.4 \times 10^6$ |
| | | 10 | $5.0 \times 10^7$ | $1.7 \times 10^6$ |
| | | 15 | $5.5 \times 10^7$ | $1.6 \times 10^6$ |
| $2.6 \times 10^9$ | 7:1 | 2 | $5.5 \times 10^7$ | $3.4 \times 10^6$ |
| | | 5 | $4.7 \times 10^7$ | $2.2 \times 10^6$ |
| | | 10 | $4.4 \times 10^7$ | $1.5 \times 10^6$ |
| | | 15 | $4.3 \times 10^7$ | $1.2 \times 10^6$ |

Data plotted as adsorption curve (FIGURE 4).

TABLE VI. Adsorption of Virus at Varying Ages of Cell Culture -- Infected Center and Unadsorbed Phage Assays

| | | | PFU per ml | |
|---|---|---|---|---|
| Age of Culture (hours) | Cell density bacteria/ml | Time (Min.) | Infected Centers | Unadsorbed Phage |
| 1.6 | $2.5 \times 10^8$ | 2 | $1.1 \times 10^7$ | $4.4 \times 10^5$ |
| | | 5 | $9.7 \times 10^6$ | $1.4 \times 10^5$ |
| | | 10 | $9.0 \times 10^6$ | $1.1 \times 10^5$ |
| | | 15 | $2.1 \times 10^7$ | $1.3 \times 10^7$ |
| 2.5 | $2.7 \times 10^8$ | 2 | $9.0 \times 10^6$ | $7.3 \times 10^5$ |
| | | 5 | $9.6 \times 10^6$ | $3.6 \times 10^5$ |
| | | 10 | $9.2 \times 10^6$ | $1.5 \times 10^5$ |
| | | 15 | $2.4 \times 10^7$ | $2.3 \times 10^7$ |
| 12 | $2.6 \times 10^8$ | 2 | $5.3 \times 10^6$ | $6.3 \times 10^5$ |
| | | 5 | $5.5 \times 10^6$ | $4.6 \times 10^5$ |
| | | 10 | $7.2 \times 10^6$ | $2.6 \times 10^5$ |
| | | 15 | $7.7 \times 10^6$ | $4.0 \times 10^5$ |

Data plotted as adsorption curve (FIGURE 5).

TABLE VII. Adsorption of Virus at Varying Virus Concentration -- Infected Center and Unadsorbed Phage Assays

| Virus Concentration PFU per ml | Dilution | Time (Min.) | PFU per ml Infected Centers | PFU per ml Unadsorbed Phage |
|---|---|---|---|---|
| $4.4 \times 10^7$ | 0 | 2 | $8.0 \times 10^7$ | $6.6 \times 10^6$ |
| | | 5 | $8.1 \times 10^7$ | $3.2 \times 10^6$ |
| | | 10 | $7.8 \times 10^7$ | $2.2 \times 10^6$ |
| | | 15 | $9.0 \times 10^7$ | $1.9 \times 10^6$ |
| $4.2 \times 10^5$ | 1:200 | 2 | $8.8 \times 10^5$ | $4.0 \times 10^4$ |
| | | 5 | $8.6 \times 10^5$ | $3.0 \times 10^4$ |
| | | 10 | $9.3 \times 10^5$ | $2.0 \times 10^4$ |
| | | 15 | $9.6 \times 10^5$ | $3.5 \times 10^4$ |

Data plotted as adsorption curve (FIGURE 6).

## SERUM NEUTRALIZATION

### Discussion, Results

Phage specificity of an antiserum provides one useful index for comparing the relationships between and among phages. Such an index is expressed in terms of K-values which represent neutralization or inactivation potency of serum samples. A series of serum titrations were conducted with two different antisera against a set of T-phages to observe rates of inactivation by varying serum dilutions at different time intervals.

Phage-antiserum mixtures were sampled at various times and assayed for survivors. These results are shown in TABLE I for anti-T2 serum, and TABLE II for anti-T1 serum. It is evident that anti-T2 has great potency against T2, showing complete neutralization by 10 minutes at a 1:100 dilution. Noticeable potency is still retained at a 1:1000 dilution. When titrated against another T-even phage, T4, significant inactivation is observable at a 1:100 dilution, with lesser effectiveness at 1:1000.

TABLE II indicates the result obtained for the titration with anti-T1 serum. These results are less striking than those seen with anti-T2. Apparent neutralization is observable at 1:100 dilution, but proves far less effective at 1:1000. When titrated against a T-even phage, T4, no inactivation was demonstrable, even at a high serum concentration of 1:10.

Neutralization curves were constructed from the titration data. FIGURES 1 and 2 depict the rates of neutralization at various time intervals for anti-T2 and anti-T1 sera. It may be seen that the inactivation becomes more apparent in the titration of serum against the homologous phage than with the heterologous phage. Phage controls without antiserum were also prepared and indicated no apparent neutralization.

If two phages are serologically related, then the antiserum of one phage will effectively neutralize the other. The K-values were determined for each set of titrations from the neutralization curves plotted, and served as basis for comparing relatedness (TABLE III). K values for inactivation of T2 and T4 by anti-T2 were within the 50-150 range. For T1, however, K values were below 10. Phages T2 and T4 appear to belong in the same serological group, while T1 is in another group. These observations are comparable to those previously reported (Adams, 1950).

The neutralization power of an antisera will vary with the serum lots. Antisera thus have different dilution ranges which give measurable activity. As observed, anti-T1 serum had a smaller activity range than did the anti-T2 which demonstrated potency at dilutions above 1:1000. Testing various serum dilutions have important applications, especially in determining the appropriate dilution for use in elimination of excess free phages in various experiments monitoring virus adsorption or growth.

TABLE I. Varying strengths of phage-specific antiserum - anti-T2

| | | | PFU per ml | |
|---|---|---|---|---|
| Antiserum | Dilution | Time | T2 | T4 |
| Anti-T2 | 1:100 | 0 min. | $6.5 \times 10^6$ | $3.0 \times 10^6$ |
| | | 3 min. | $1.6 \times 10^5$ | $1.5 \times 10^6$ |
| | | 10 min. | 0 | $1.2 \times 10^5$ |
| | | 30 min. | 0 | 0 |
| | *1:1000 | 0 min. | $7.0 \times 10^6$ | $3.2 \times 10^6$ |
| | | 3 min. | $3.1 \times 10^6$ | $3.4 \times 10^6$ |
| | | 10 min. | $1.7 \times 10^6$ | $2.4 \times 10^6$ |
| | | 30 min. | $1.7 \times 10^5$ | $1.5 \times 10^6$ |

*Data plotted for neutralization curve (Figure 1).

TABLE II. Varying strengths of phage-specific antiserum - anti-Tl

| Antiserum | Dilution | Time | PFU per ml<br>T2 | <br>T4 |
|---|---|---|---|---|
| Anti-Tl | 1:10 | 0 min. | | $1.3 \times 10^7$ |
| | | 3 min. | | $1.3 \times 10^7$ |
| | | 30 min. | | $1.2 \times 10^7$ |
| | *1:100 | 0 min. | $2.2 \times 10^6$ | $1.2 \times 10^7$ |
| | | 3 min. | $2.0 \times 10^6$ | $1.2 \times 10^7$ |
| | | 30 min. | $6.5 \times 10^5$ | $1.3 \times 10^7$ |
| | 1:1000 | 0 min. | $2.1 \times 10^6$ | |
| | | 3 min. | $1.9 \times 10^6$ | |
| | | 30 min. | $1.3 \times 10^6$ | |

*Data plotted for neutralization curve (Figure 2).

TABLE III. K-values for Anti-T2 and Anti-T1 antisera

| Antiserum | Dilution | Phage | K-value/min |
|---|---|---|---|
| anti-T2 | 1:1000 | T2 | 133.4 |
| | 1:1000 | T4 | 69.0 |
| anti-T1 | 1:100 | T1 | 5.52 |
| | 1:100 | T4 | - |

K-values determined for t = 10 min.

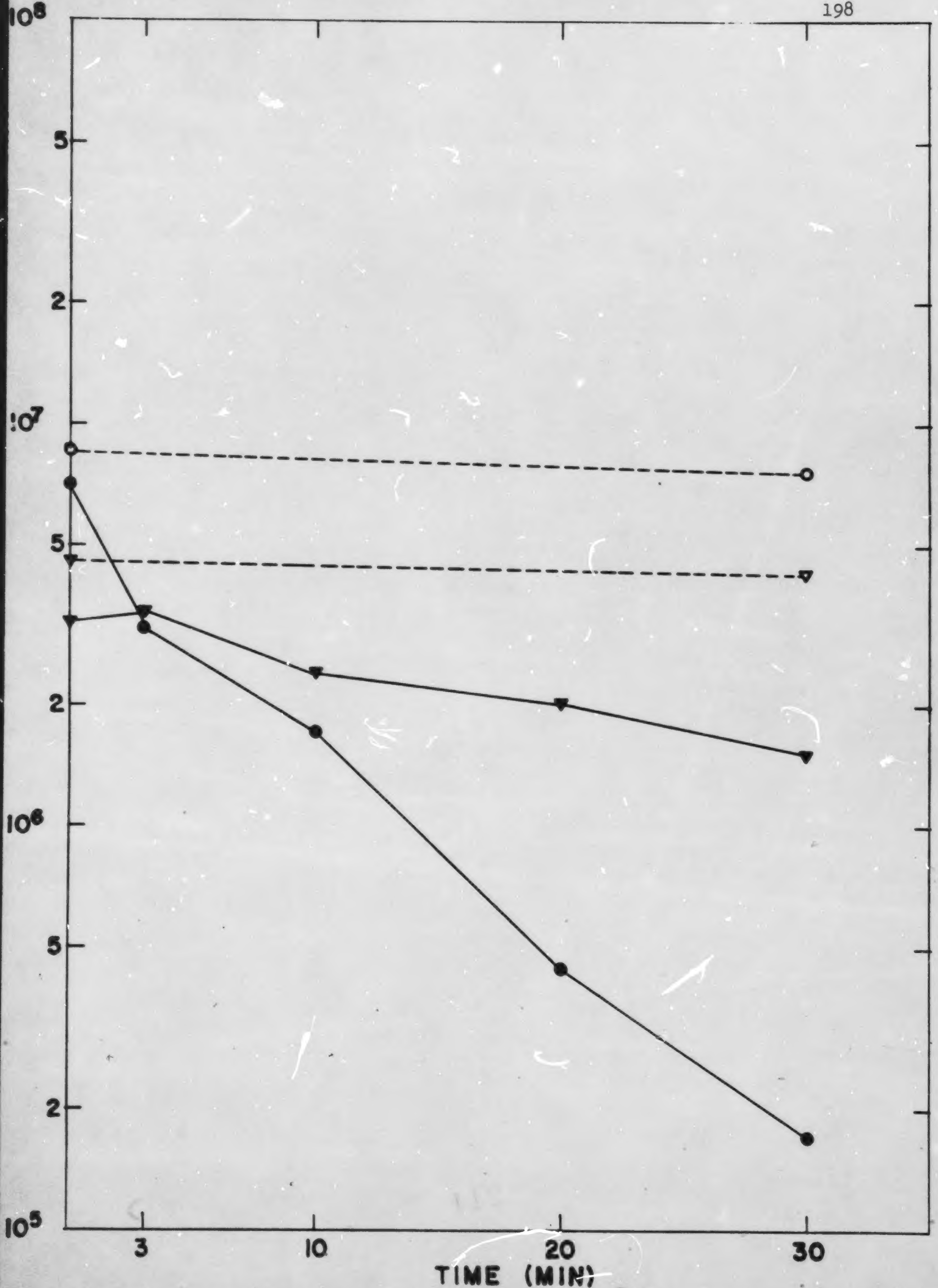
Neutralizing activity of T2 phage antiserum.

Figure 1.

A 1.8 ml serum sample of anti-T2 diluted 1:1000 was mixed with a 0.2 ml volume of T2 phage at $5.0 \times 10^6$ PFU per ml. One-tenth of a milliliter samples were taken, diluted, and assayed for phage survivors (●).

This was repeated with 0.2 ml of T4 phage at $4.5 \times 10^6$ PFU per ml. Both phage-antisera mixtures were incubated and maintained at 37°C during all samplings (▼).

Control cultures of phage in diluent without antisera were also assayed (o, T2; ∇, T4).


10^8
5
2
10^7
5
2
10^6
5
2
10^5
3
10
20
30
TIME (MIN)

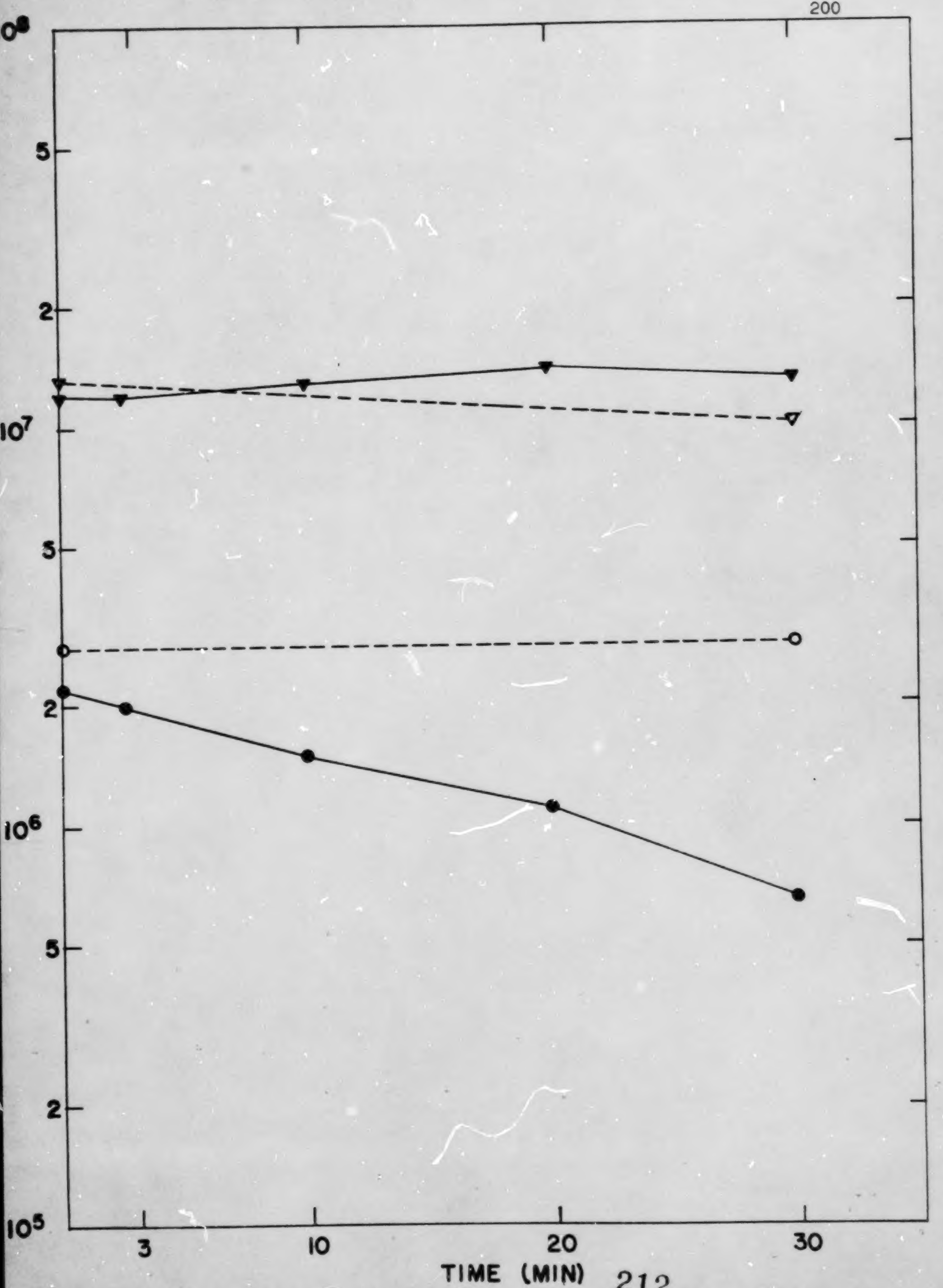
Neutralizing activity of T1 phage antiserum.

FIGURE 2.

A 1.8 ml serum sample of anti-T1 diluted 1:100 was incubated with 0.2 ml of T1 phage at 2.8 × $10^6$ PFU per ml. One-tenth milliliter samples were assayed for survivors (●).

This was conducted for anti-T1 diluted 1:10 with 0.2 ml of T4 phage at 3.0 × $10^6$ PFU per ml and 0.1 ml samples titrated (▼).

Control cultures with no antisera were included (○, T1; ∇, T4).



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## PHAGE-RESISTANT MUTANTS

### Discussion, Results

A. Phage-resistant mutants have been described to be detectable at low frequency levels. Such colonies may be isolated and identified from broth cultures. Studies were conducted to investigate the occurrence of mutations to the T-phages under varying experimental conditions and to identify the common types present (TABLE I).

Under normal growing conditions, it is possible to isolate resistant colonies to the different phages. Representative colonies were observed for characteristic colonial and cell morphology. These were found similar to the parent strain with slight minor variations in size and surface features. All resistant cells determined by gram stain for purity check were observed to be gram-negative, small, short rods, as were the parental cells. A number of colonies were selected and tested for phage resistance. Most appear to possess different combinations of multiple resistance, especially to T4 and T7. Some single resistance colonies were found for T4.

An alternative method employing a chemical mutagenic agent, N-methyl-N'-nitro-N-nitrosoguanidine, was evaluated for its effects in increasing mutant yields. A young E. coli β culture was centrifuged, resuspended into a volume of medium containing the agent, and incubated at 37 C for 5 minutes before exposure to phage. The number of resistant colonies was greatly enhanced. Representative colonies tested indicated that the isolates were mainly B/4,7. These results, however, reflected only a very small portion of the colonies tested, and isolating other combinations would require the survey of the remaining colonies.

Ultra-violet irradiation has also been known to produce mutagenic effects. Application of this agent was attempted and compared. It appeared unsuccessful under these experimental conditions, producing no significant increase in the number of resistant colonies over that of the normal culture.

The occurrence of B/2 mutants have been described as a rare event ($10^{-10}$). Under each of the varying experimental conditions already employed, all results obtained were negative. In an effort to increase frequency of isolation, another method involved the concentration of T2-infected cultures and plating increased numbers of infected centers. No B/2 mutants could be detected.

For study purposes, it is adequate that isolation of phage-resistant mutants may be accomplished successfully from normal, growing cultures. Variations may employ the use of different induction agents. Nitrosoguanidine has been demonstrated to be an effective agent, although its carcinogenic property presents a hazard and should limit its use in student laboratories. Ultra-violet irradiation may prove more applicable if appropriate experimental design can be devised. As for the isolation of a rare mutant, this may require the plating of more than 1000-fold increases in the number of infected cells and the continuous screening of resistant colonies, to isolate a resistant mutant strain.

TABLE I.

Nitrosoguanidine was incorporated in a volume of medium containing resuspended culture of 2.5 hours E. coli β. The culture was incubated with the agent for 5 minutes at 37 C prior to phage infection.

Five ml samples of a 2.5 hour bacterial culture was exposed to a germicidal UV lamp for 10 and 15 seconds from a distance of 20 cm and kept in foiled-covered tubes during phage infection and plating.

A normal culture was prepared as a young 2.5 hour culture and plated in phage excess.

All isolates were tested for purity by the Gram stain, restreaked for single colonies, and observed for distinct colonial morphology.

The number of resistant colonies were cumulative results obtained from the 5 replicate plates. The type of mutants observed were indicative of the isolates identified from 5 representative colonies selected for each study.

TABLE I. Effects of Inducing Agents on *E. coli* B

| Inducing Agent | No. of Resistant Colonies | Mutant Type Isolated |
|---|---|---|
| Nitrosoguanidine | 265 | B/4,7 |
| Ultra-violet | 7 | B/4,7<br>B/4 |
| Normal culture | 79 | B/4<br>B/1,4<br>B/4,7<br>B/1,4,7 |

## PHAGE-RESISTANT MUTANTS

### Discussion, Results

B. Host resistance has already been demonstrated for *E. coli* β to the various T-phages. The basis for this resistance was investigated by monitoring evidence for phage adsorption and virus yields of a phage-resistant mutant of T2 (obtained from Dr. S. E. Luria's collection) with that of the sensitive parent.

The growth rate of the mutant strain was determined initially to standardize its growth with that of the parent strain. As described (5) growth of the two strains does not differ appreciably. The β/2 culture was observed at varying time intervals for a 24-hour period, growth being monitored by viable counts and optical density determinations and compared to the parent culture (FIGURES 1 and 2). Comparisons were made up to the 6-hour growth period, particularly noting cell concentrations at 2 to 2.5 hours, which would represent the experimental cultures to be utilized. Results appear similar to the parent culture. The overall growth of the mutant, however, was not comparable under these experimental conditions final and cell densities were lower. It should be considered again, that this may reflect the influence of various physiological and other environmental factors on bacterial growth.

With these observations, young cultures of the mutant strain as well as of the parent strain, both similar in age and cell concentration, may be used in parallel experiments testing host resistance.

A designated amount of phage was incubated with each bacterial culture (2.5 hours) in the presence of KCN and assayed for virus yields after disruption with chloroform. KCN is described as a metabolic inhibitor which interfered with the progress and formation of virus progeny (1, 3). Its action is observed as a resulting loss of infectious centers early in the latent period (3).

Sampling was taken during the latent period (15 minutes) of a T2-infected E. coli β parental strain incubated in the presence of KCN. The assay results (TABLE I) were compared to a control culture incubated in the absence of KCN. The slightly higher phage numbers may be due to early release of phages from some cells, as chloroform was added to inactivate infected cells by premature lysis. Samples were also assayed at 30 minutes, after allowing intracellular multiplication to occur. The control culture exhibited normal viral growth as evidenced by increase phage yields. The phage-infected culture with KCN at 30 minutes demonstrated yields comparable to those obtained by the 15 minute assay. This finding supports the observation of early loss of infected centers by the action of KCN which appeared to prohibit further phage development into mature progeny, as evidenced by no increase in either sample of phage numbers with premature lysis.

This experimental procedure was used with a β/2 mutant culture. If one assumes that host resistance was attributable to alterations of phage development within the host cell, then it would be expected that phage numbers would decrease significantly due to adsorption. This was not observed, as assay samples taken at 15 and 30 minutes reveal that virus yields did not differ appreciably in the presence or absence of KCN (TABLE I). It appears that host resistance results in the failure of phage to adsorb to host cells.

Controls containing KCN with the bacterial culture or phage sample indicated slight cell killing and little or no inactivation of free phage particles.

These experimental observations and results appear to support the view that one basis of host resistance to bacteriophage infection involves some alteration of the host and, as discussed previously, particularly changes at specific sites of attachment on the cell surface.

TABLE I. Host Resistance to Bacteriophage T2

| Experimental Assay | Time (min.) | PFU per ml |
|---|---|---|
| *E. coli* β + T2 | 15' | $1.07 \times 10^5$ |
| *E. coli* β + T2 + KCN | 15' | $4.5 \times 10^4$ |
| β/2 mutant + T2 | 15' | $3.3 \times 10^6$ |
| β/2 mutant + T2 + KCN | 15' | $4.75 \times 10^6$ |
| *E. coli* β + T2 | 30' | $1.02 \times 10^7$ |
| *E. coli* β + T2 + KCN | 30' | $3.9 \times 10^4$ |
| β/2 mutant + T2 | 30' | $3.6 \times 10^6$ |
| β/2 mutant + T2 + KCN | 30' | $4.65 \times 10^6$ |
| Controls: | | |
| Phage T2 | | $3.9 \times 10^6$ |
| Phage T2 + KCN | | $5.4 \times 10^6$ |
| *E. coli* β | | $8.0 \times 10^8$ |
| *E. coli* β + KCN | | $4.3 \times 10^8$ |
| β/2 mutant | | $3.7 \times 10^8$ |
| β/2 mutant + KCN | | $3.6 \times 10^8$ |

## PHAGE-RESISTANT MUTANTS

### Discussion, Results

C. Phage titrations were carried out employing a mixed indicator assay composed of equal amounts of the sensitive parent *E. coli* β strain and the resistant mutant β/2 strain (obtained from Dr. S. E. Luria's collection for this particular experiment). The β/2 culture was observed to be sensitive to T1, T4, and T7 phages, while completely resistant to the T2 phage.

The data in TABLE I indicate sensitivity of the parent culture to both T2 and T4, as expected. The β/2 mutant is only sensitive to T4 and resistant to T2, as evidenced by the presence or absence of plaque formation. Using mixed indicators in equal portions, phage titers were comparable. The main distinguishing characteristic in this assay is found in plaque morphology. Sensitive strain indicators reveal clear plaques. Mixed indicators, as in the case for T2, demonstrate turbid plaques, supporting the observation that the sensitive cells are lysed by the phage with continued growth of the resistant cells.

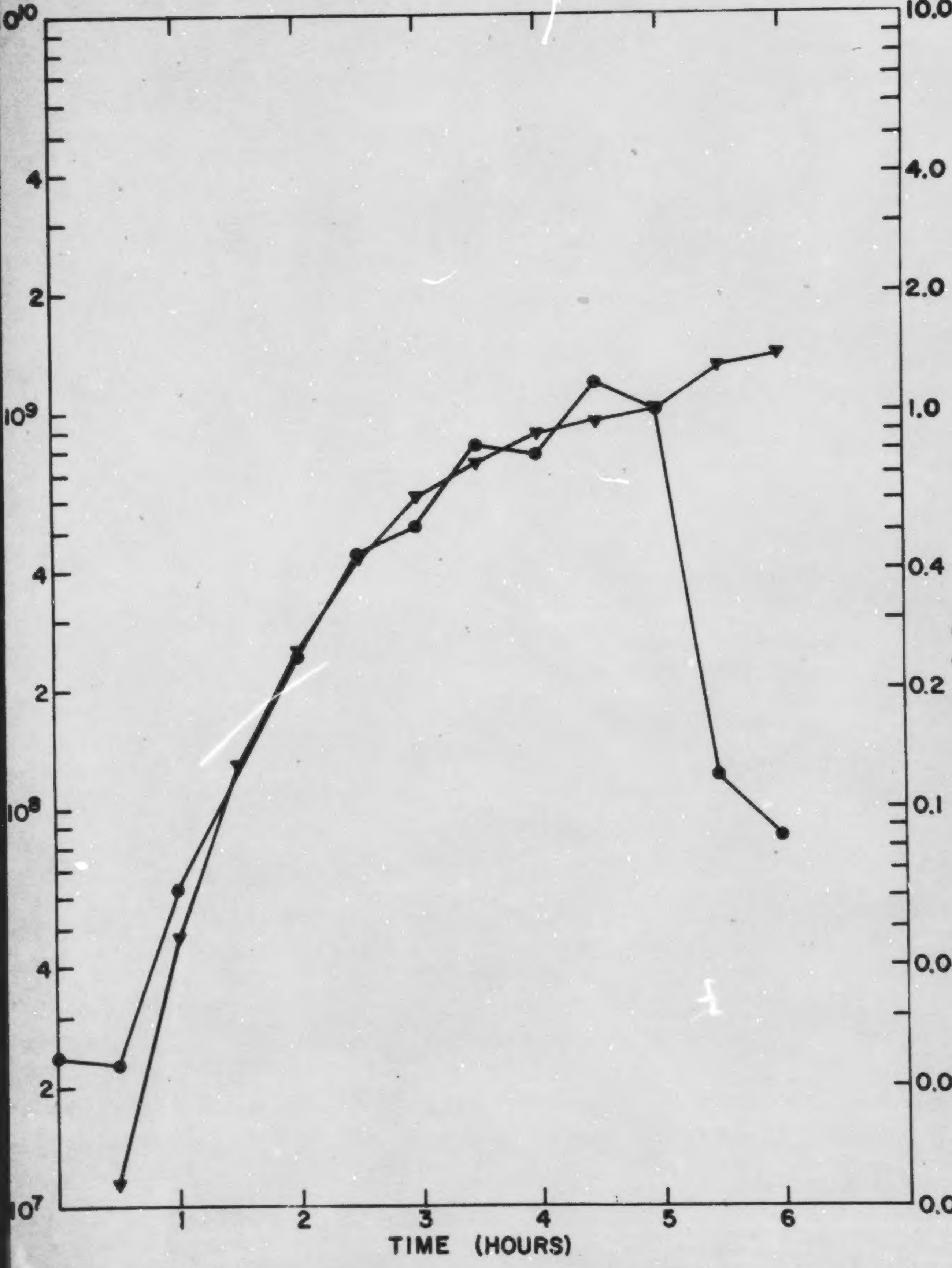
Fig. 1. Growth curve of T2 phage mutant of *E. coli* β.

Fig. 2. Growth curve of *E. coli* β.

Figures 1-2.

Both normal and mutant cultures were prepared by 1:200 dilution of an overnight culture into fresh, prewarmed media (TPO4). Incubation was made at 37 C. Samples of 4.5 mls of each culture were taken at the indicated time intervals and determined for optical density at a wavelength 550 mμ. Samples of 0.5 ml of each culture were taken at different times, diluted, and assayed for viable cell counts by the spread plate method.

● viable cells
▼ optical density


10¹⁰
4
2
10⁹
4
2
10⁸
4
2
10⁷
10.0
4.0
2.0
1.0
0.4
0.2
0.1
0.04
0.02
0.01
O. D.550
1
2
3
4
5
6
TIME (HOURS)

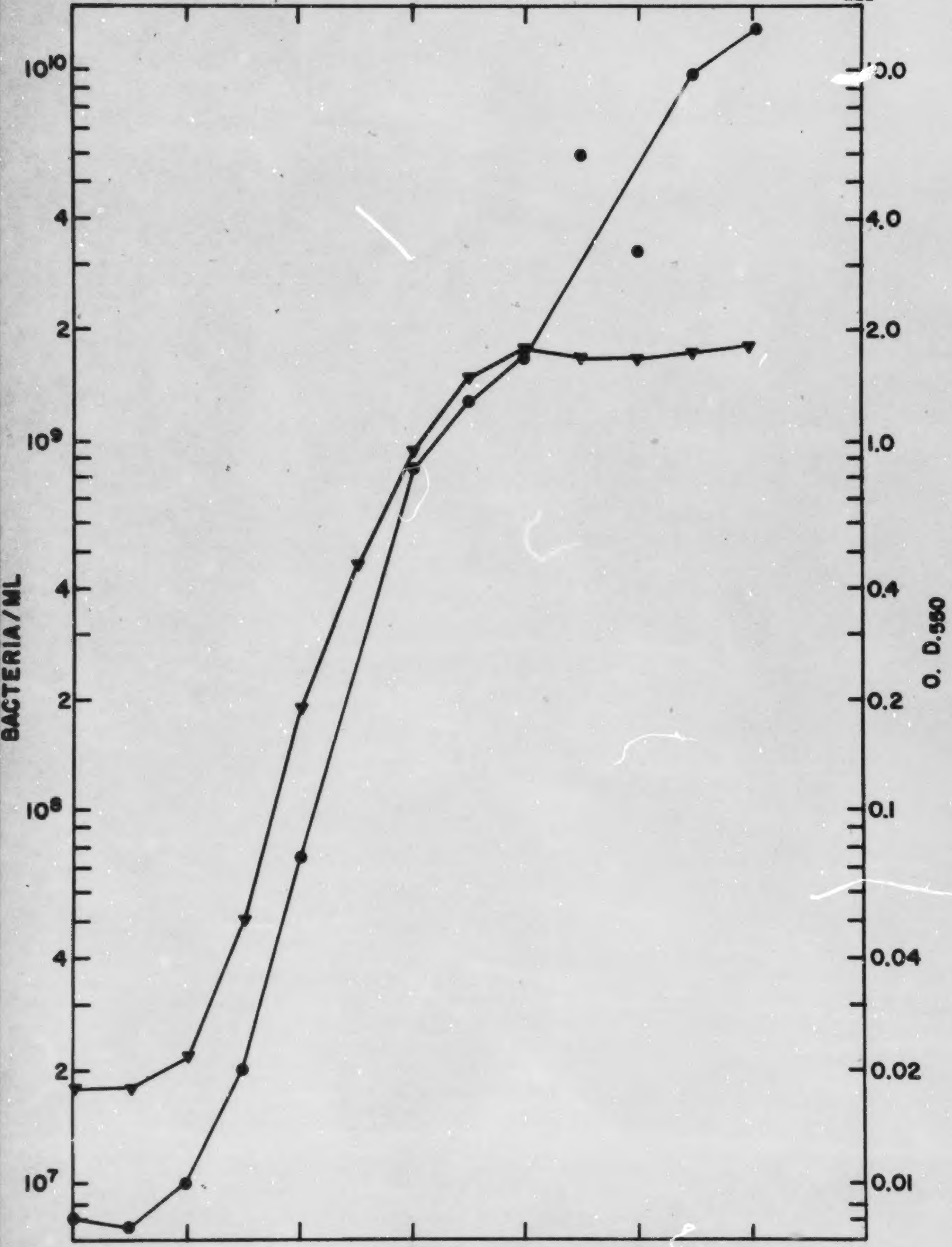

10^10
4
2
10^9
4
2
10^8
4
2
10^7
BACTERIA/ML
10.0
4.0
2.0
1.0
0.4
0.2
0.1
0.04
0.02
0.01
O. D.550
1
2
3
4
5
6
TIME (HOURS)

TABLE I. Plating Mixed Indicators with Phages T2 and T4.

| Indicator Culture | Phage | PFU per ml |
|---|---|---|
| *E. coli* β | T2 | $5.5 \times 10^{10}$ |
| β/2 mutant | T2 | 0 |
| *E. coli* β + β/2* | T2 | $5.7 \times 10^{10}$ |
| *E. coli* β + β/2** | T2 | $5.5 \times 10^{10}$ |
| *E. coli* β | T4 | $8.4 \times 10^{9}$ |
| β/2 mutant | T4 | $7.3 \times 10^{9}$ |
| *E. coli* β +β/2* | T4 | $7.7 \times 10^{9}$ |

* Equal portions (0.05 ml) of both cultures were inoculated.

** Equal portions (0.10 ml) of both cultures were inoculated.

# LYSOGENY

## Discussion, Results

In a lysogenic culture, it is possible to detect a low background phage level which results from occasional spontaneous cell lysis. Increase in phage production in such a culture may be accomplished by the use of various experimental inducers that enable the prophage to enter the lytic cycle. One example is the use of ultraviolet irradiation. This agent was used with Escherichia coli K12 ($\lambda$) and the culture studied for UV effects in enhancing phage numbers.

TABLE I indicates results obtained when 5 ml samples of E. coli K12 ($\lambda$) were subjected to varying lengths of exposure to UV light. After incubation for cell lysis, the cultures were titrated with an indicator organism to detect virus particles. A low background level was detected in the non-irradiated control. However, increases of greater than two-fold above the control level were observed after a 3 second treatment. This was noted for the other exposures employed. The 5 second time was selected as the more appropriate exposure for use in subsequent induction experiments.

The presence of phage may also be evidenced by cell lysis, as determined through optical density measurmments. This was monitored in cultures irradiated for 5 and 10 seconds (FIGURE 1). Control and irradiated curve profiles showed noticeable differences in optical density units, hence, cell lysis and phage production. It has been said that the proportion of phage-producing bacteria will be a function of different variables. For UV light, consideration is given to the distance, wavelength, and exposure time, in addition to the type and quality of media employed.

Investigations were carried out to determine the number of surviving bacteria as compared to the numbers of phage-producing bacteria. Samples taken from the 5 second irradiated cultures during the incubation period were diluted and titrated simultaneously for viable cells and phage numbers. These results have been plotted and shown in FIGURE 2. Control cultures continue cell growth with time and a low level of free phage was detectable. Irradiated cultures reflected a decrease in cell numbers, mainly due to cell lysis and cell killing by UV, with a corresponding increase in phage numbers that eventually reach a maximum and then decrease to a rate affected by the UV dosage on the bacterial culture.

In a lysogenic culture, it has been said that no infection of lysogenic cells will occur, as was evident in the T-phages. This can be demonstrated, as presented in the experimental design described, by incubation of the supernatant fluid containing infectious phage with the non-irradiated culture. Titration for viable cells should reflect significant changes in the cell concentration from the original culture if cells were sensitive to phage infection. This was not observed and is attributed to an "immunity factor," protecting cells from homologous lysogenic or very closely-related phages.

Lysogeny has been demonstrated in several bacterial systems as the unique potential by which cells have the capability to produce infectious phages under the appropriate conditions. As described, this phenomenon has already provided areas of extensive study, especially in experimental induction and genetic analyses.

Optical density of UV-induced lysogenic cultures of E. coli K12 (λ).

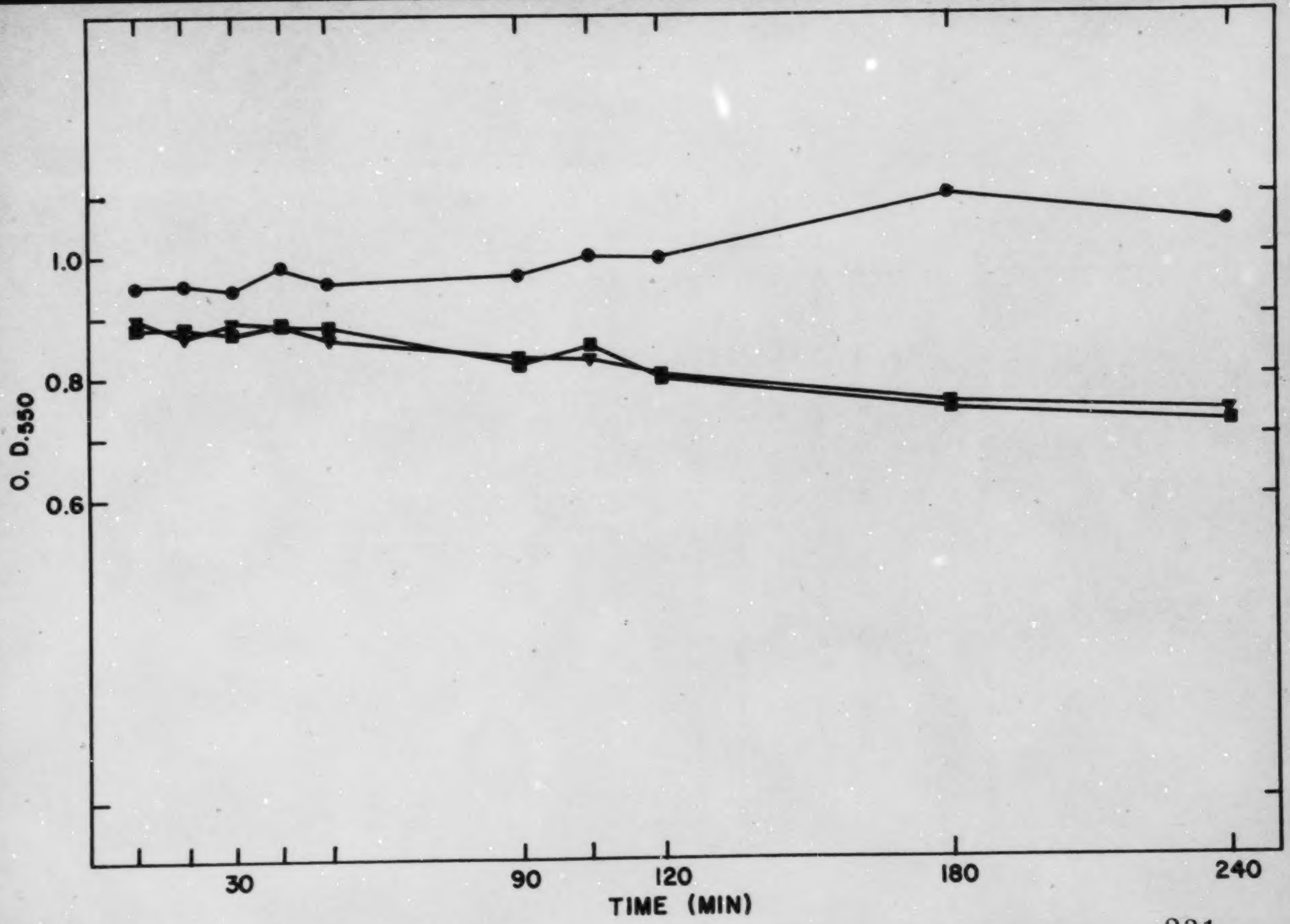
Figure 1.

Five ml aliquots of a young lysogenic culture of E. coli K12 (λ) were UV-irradiated and incubated at 37 C. Optical density readings at a wavelength of 550 mμ were made at indicated time intervals for cell killing by induced prophages,

■ A, 10 sec UV-irradiated culture

▼ B, 5 sec UV-irradiated culture

● C, control culture, no irradiation


O. D.550
1.0
0.8
0.6
30
90
120
180
240
TIME (MIN)

Virus titration and colony formation of UV-induced lysogenic cultures of *E. coli* K12 (λ).

Figure 2.

Lysogenic culture of *E. coli* K12 (λ) was prepared by a 1:200 dilution of an overnight culture into fresh, prewarmed broth (TPO4) at 37 C and grown to appropriate density. Cells were pelleted and resuspended in synthetic M9 media. Culture were UV-irradiated and at indicated time intervals sampled for viable cells and presence of infectious phage,

A, 5 sec UV-irradiated culture -- viable cells
phage (● cells, o phage)

B, non-irradiated control cuture -- viable cells
phage (▼ cells, ▽ phage)

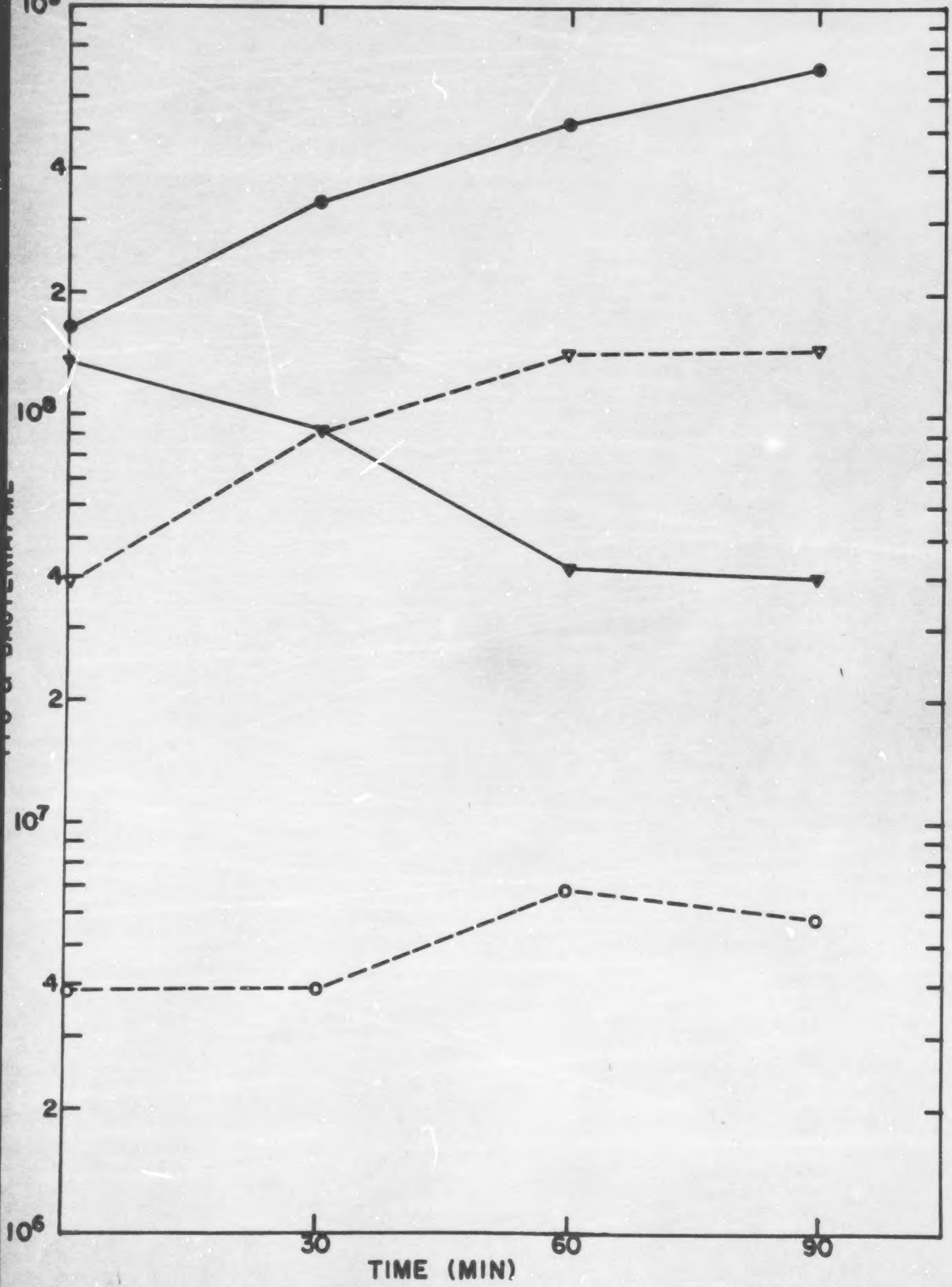

10⁹
4
2
10⁸
4
2
10⁷
4
2
10⁶
30
60
90
TIME (MIN)

TABLE I. Induction of *E. coli* K12 (λ) with varying exposures of UV light

| UV exposure (sec.) | PFU per ml |
|---|---|
| 0 | $7.3 \times 10^3$ |
| 3 | $1.4 \times 10^6$ |
| 5 | $2.25 \times 10^6$ |
| 10 | $2.3 \times 10^6$ |
| 25 | $2.8 \times 10^6$ |

TABLE II. Optical Density Measurements of a Lysogenic Culture of *Escherichia coli* K12

| Time (min.) | Control | UV Culture (5 sec.) | UV Culture (10 sec.) |
|---|---|---|---|
| 10 | 0.95 | 0.9 | 0.9 |
| 20 | 0.96 | 0.87 | 0.88 |
| 30 | 0.94 | 0.88 | 0.87 |
| 40 | 0.99 | 0.89 | 0.89 |
| 50 | 0.96 | 0.87 | 0.87 |
| 90 | 0.97 | 0.83 | 0.83 |
| 105 | 1.0 | 0.85 | 0.85 |
| 120 | 1.0 | 0.81 | 0.81 |
| 150 | 1.1 | 0.75 | 0.75 |
| 240 | 1.1 | 0.73 | 0.73 |

Data plotted as optical density curve (FIGURE 1).

TABLE III. Viable Count and Phage Assay of Lysogenic Culture of *Escherichia coli* K12

| Assay | Sampling time (min.) | Bacteria/ml | PFU/ml |
|---|---|---|---|
| *E. coli* K12 | 0 | $1.68 \times 10^8$ | $3.89 \times 10^6$ |
| | 30 | $3.4 \times 10^8$ | $3.91 \times 10^6$ |
| | 60 | $5.3 \times 10^8$ | $6.8 \times 10^6$ |
| | 90 | $7.2 \times 10^8$ | $5.8 \times 10^6$ |
| *E. coli* K12 | 0 | $1.36 \times 10^8$ | $4.3 \times 10^7$ |
| (irradiated for 5 sec.) | 30 | $9.3 \times 10^7$ | $9.5 \times 10^7$ |
| | 60 | $4.2 \times 10^7$ | $1.48 \times 10^8$ |
| | 90 | $4.0 \times 10^7$ | $1.51 \times 10^8$ |

Data plotted as titration curve (FIGURE 2).

## T4 MORPHOGENESIS: IN VITRO COMPLEMENTATION

### Discussion, Results

*In vitro* complementation experiments were attempted with cell extracts with two different tail fiber mutants (T4am N52 and T4am B252) with gene defects at gene 37 and 35, respectively. Cell extracts were prepared as described with the nonpermissive host, *E. coli* β (su⁻). Assay of the reaction mixture was performed using the permissive host, *E. coli* CR63.

Several trials were conducted with different cell extract preparations. Incubation of the control extracts alone demonstrated no increase in active phage numbers. Generally, little success was observed with the reaction mixture, with no significant increase above the level of the controls obtained (TABLE I). This suggests that in our hands, expected complementation did not take place between the two defective mutants.

In addition, complementation tests were conducted with tail-fiberless particles (T4am X4E, a multiple amber mutant with several mutations: B25, A455, B252, N52, B280, and B262 which causes tail fiber genes 34, 35, 37, and 38 to be defective) and a head protein extract (T4am B17, defective in gene 23 that controls head morphogenesis). As reported (1), the number of active phage produced should be proportional to the number of tail-fiberless particles added to the extract. No increase in phage titer was indicated by incubation of the extract or particles alone. Although the number of particles was not determined here, the kinetics of infectivity, or active phage production, remained level throughout the incubating period and reflected no positive activation of the tail-fiberless particles (TABLE II).

It is apparent that the complete inactivity of the various extract combinations indicates that the complementation reactions did not proceed effectively as hoped under the present experimental conditions described. These experiments have demonstrated little success here. It is difficult to ascertain the various factors involved in these problems. Among problems to be considered, the nature and optimal condition for cell extract preparations as well as the character of the amber mutant stocks employed are suggested. Further, whether they are truly defective as reported and produce an accumulation of the incomplete phage components is not known for our stocks. It is necessary, therefore, to reevaluate in detail the procedures and especially the materials described here and to make further modifications before conducting other extract complementation tests.

TABLE I. Extract Complementation of Tail Fiber Mutants

| Experiment Number | Infected-cell Extracts | Time (Min.) | PFU per ml |
|---|---|---|---|
| 1 | A | 0 | $1.5 \times 10^4$ |
| | B | | $3.5 \times 10^7$ |
| | AB | | $1.1 \times 10^7$ |
| | A | 90 | $1.5 \times 10^4$ |
| | B | | $3.4 \times 10^7$ |
| | AB | | $1.0 \times 10^7$ |
| 2 | A | 0 | $7.8 \times 10^6$ |
| | B | | $3.0 \times 10^8$ |
| | AB | | $1.5 \times 10^8$ |
| | A | 90 | $4.5 \times 10^6$ |
| | B | | $2.9 \times 10^8$ |
| | AB | | $7.0 \times 10^7$ |

A, infected-cell extract by amN52, control

B, infected-cell extract by amB252, control

AB, combination of both extracts

TABLE II. Extractive Complementation of Tail-fiberless Particles and Head Protein Defective Extract

| Experiment Number | Infected-cell Extracts | Time (Min.) | PFU per ml |
|---|---|---|---|
| 1 | C | 0 | $1.6 \times 10^3$ |
| | D | | $6.0 \times 10^3$ |
| | CD | | $1.2 \times 10^4$ |
| | CD | 10 | $1.1 \times 10^4$ |
| | CD | 30 | $2.5 \times 10^3$ |
| | CD | 45 | $3.5 \times 10^3$ |
| | CD | 60 | $2.5 \times 10^3$ |
| | C | 60 | $1.9 \times 10^3$ |
| | D | 60 | $2.4 \times 10^3$ |
| 2 | C | 0 | $4.3 \times 10^7$ |
| | D | | $3.8 \times 10^7$ |
| | CD | | $4.9 \times 10^7$ |
| | CD | 10 | $4.8 \times 10^7$ |
| | CD | 30 | $3.0 \times 10^7$ |
| | CD | 45 | $2.0 \times 10^7$ |
| | CD | 60 | $2.0 \times 10^7$ |
| | C | 60 | $4.1 \times 10^7$ |
| | D | | $5.6 \times 10^7$ |

C, tail fiberless particles amX4E, control

D, infected-cell extract by amB17, control

CD, combination of both extract

# ENZYME INDUCTION

## Discussion, Results

Enzyme induction poses several problems for study of enzyme activity under varying conditions. Inducer efficiency and effects of phage infection provided two areas of investigation, especially for determination of the presence of the induction phenomenon.

The assay procedure, already described, is a modification from Lederberg's original method. Certain preparations should be considered to avoid experimental problems. For example, bacterial cultures grown in a synthetic medium rather than a colored nutrient medium eliminate interference with colorimetric determination of the indicator, o-nitrophenyl. Caution should also be taken with the ONPG solution as it is relatively unstable and upon long periods of storage the appearance of a yellow color may be seen (1).

Early studies demonstrated that direct, apparent detection of the induced enzyme could be facilitated when the enzyme was liberated from whole cells by cellular disintegration. Various chemical agents, e.g., toluene, benzene, have been employed, with the observation that a more intense indicator color could be obtained. The amount of color determined represents the totality of β-galactosidase enzyme present. Some investigators have introduced the use of deoxycholate in addition to toluene. Their results indicate the possibilities of a more reproducible and accurate assay method than with toluene alone (2).

Ordinarily, β-galactosidase is inducible by its normal substrate lactose. In a number of cases, the enzyme may be induced by substances which do not act as substrates, but which are structural analogues of the substrate. One such related compound is methyl-β-D-galactoside (TMG). Enzyme activity of induced, toluenized cells was monitored by optical density measurements of ONPG (FIGURE 1). Results indicate that induction had occurred both with TMG and with lactose. Noticeable differences were observable for lactose, its efficiency as an inducer being several times greater than TMG. Thus a higher enzyme content can be expected with lactose. Under these experimental conditions, this may be attributable to the differences in inducer concentration employed. It would appear probable, however, that lactose should be equally efficient even at the lower concentration.

In the presence of an alternate substrate, e.g., glycerol, however, cells do not exhibit the ability to elicit the synthesis of β-galactosidase. Such an effect can be attributable to the difference in structural configuration of the substrate. This result is shown in FIGURE 2, in which the glycerol-induced culture indicated little significant changes in optical densities during a 24-hour period. As expected, the lactose-induced culture reflected β-galactosidase induction at an appreciable level. Since the enzyme is necessary for the utilization of the substrate, an increase in enzyme content may then result in an increase in cell growth, as the energy source is transformed into an available form for use by the cells. Increase may be implied to coincide with cell growth. Then as nutrients become exhausted, especially the concentration of the inducer, a corresponding decrease in enzyme content becomes evident.

Addition of phage prior to exposure of the inducer will also prevent the synthesis of β-galactosidase in a bacterial population. This study was conducted under conditions of lysis-inhibition, in which a high input of phage, usually greater than five phage per cell, was employed for infection, so that cell lysis would be extended for several hours (TABLE I). Results in FIGURE 1 indicates that enzyme induction had been completely arrested by T2 phage infection for a 2 hour period.

A cell contains many distinct enzyme proteins. With the induction phenomenon, the cell is shown to be endowed with the potential under appropriate conditions to produce additional enzymes.

FIGURE 1.

Kinetics of β-galactosidase induction and the effect of different inducers and phage infection.

Each culture of E. coli β grown in synthetic M9 medium and aliquots of cell pellet resuspended and treated as follows:

● A, addition of lactose (0.5%) 4 min after resuspension;

▼ B, addition of methyl-β-D-thiogalactoside (0.004 M) 4 min after resuspension;

■ C, resuspension of cells, infection with T2 phage at a multiplicity of 6-8 phage per cell, addition of lactose 4 min after infection;

▲ D, control, no inducer.

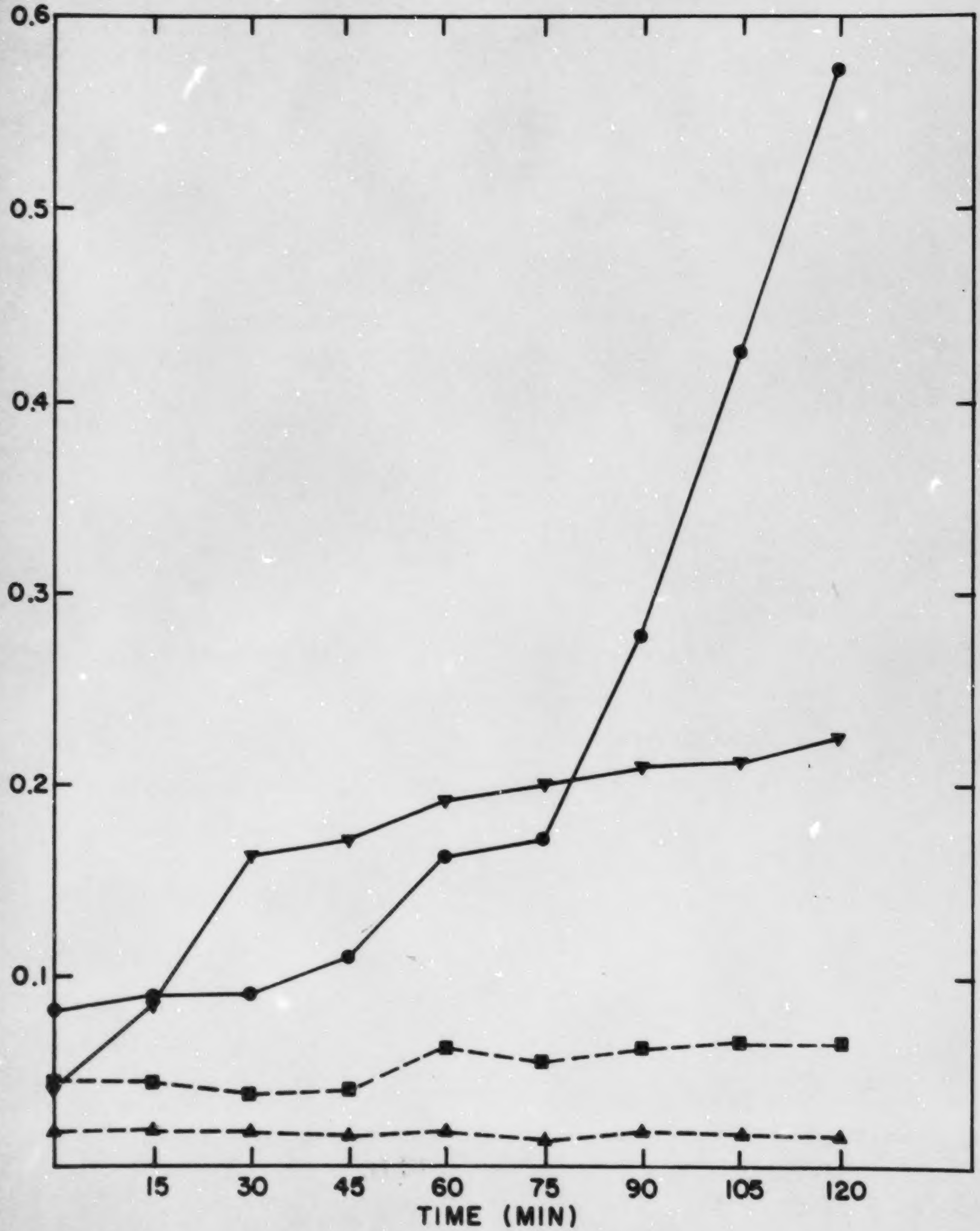

0.6
0.5
0.4
0.3
0.2
0.1
15
30
45
60
75
90
105
120
TIME (MIN)

FIGURE 2.

Kinetics of β-galactosidase by different substrates. Each culture of E. coli β grown in synthetic medium and aliquot of cell pellet resuspended and treated as follows:

- ■ A, addition of glycerol (0.5%) 4 min after resuspension;
- ▼ B, addition of lactose (0.5%) 4 min after resuspension;
- ● C, control, no inducer.

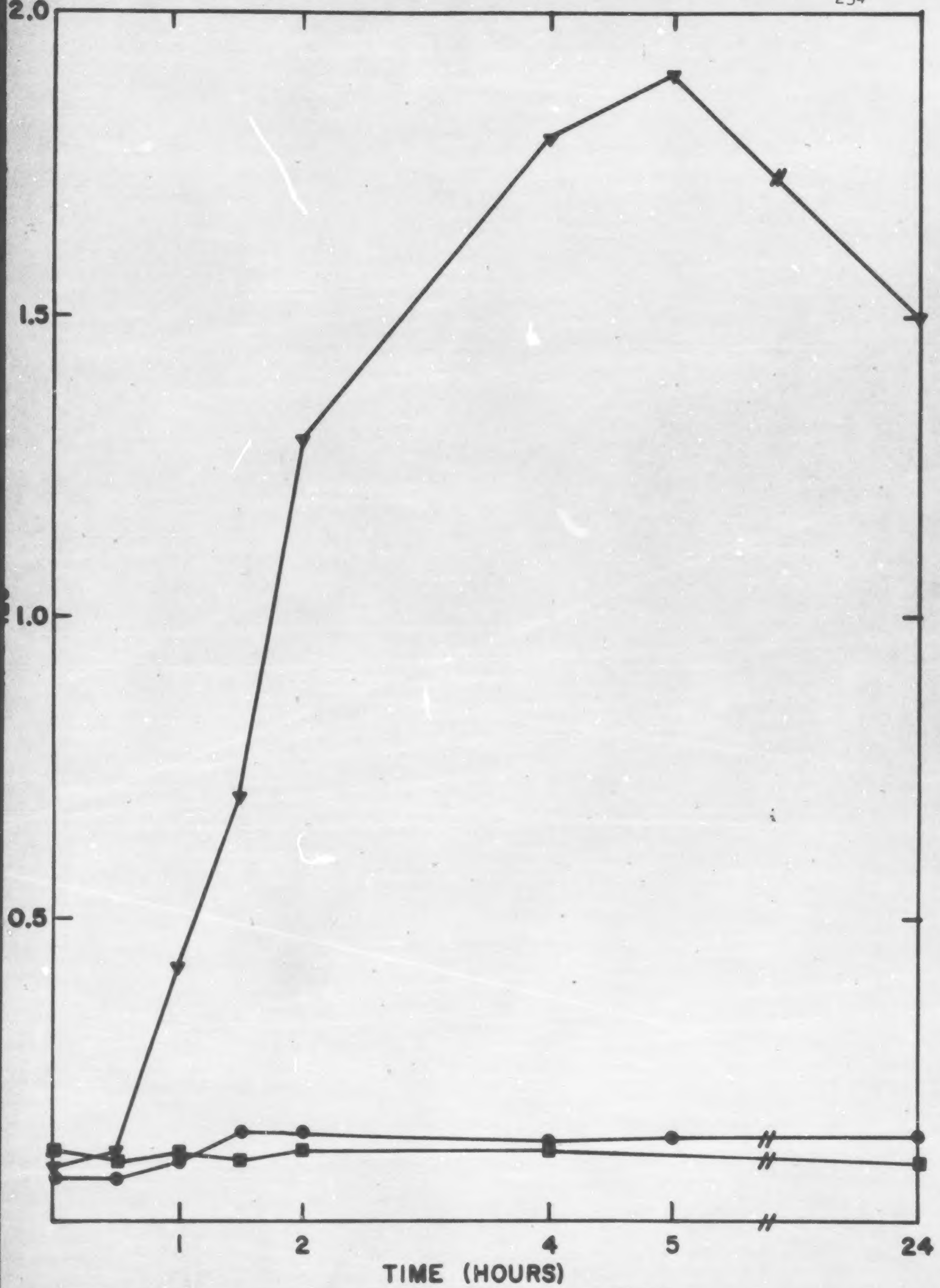

2.0
1.5
1.0
0.5
1
2
4
5
24
TIME (HOURS)

TABLE I. Lysis Inhibition of T2-infected Culture of *Escherichia coli* B

| Time (Min.) | Bacteria/ml | PFU/ml |
|---|---|---|
| 0 | $1.7 \times 10^6$ | $4.4 \times 10^7$ |
| 30 | $5.7 \times 10^5$ | $1.4 \times 10^7$ |
| 60 | $5.4 \times 10^5$ | $2.8 \times 10^7$ |
| 90 | $3.7 \times 10^5$ | $7.1 \times 10^7$ |
| 120 | $5.5 \times 10^5$ | $1.6 \times 10^8$ |

Phage infection at a multiplicity of 6 phage/cell.

# DNA EXTRACTION

## Discussion, Results

Bacterial DNA was extracted from *E. coli* β as described. Several different extractions were prepared for duplicate analyses. It is of interest to note the dramatic changes that take place during each step of the extraction.

The addition of lysozyme initiates degradation of the cell wall and lyses those cells resistant to detergent action. Treatment with SDS continues cell disruption and suppresses enzyme activity by nucleases. Lysis is completed by repeated freezing and thawing. The resulting emulsion possesses two characteristics -- a remarkable increase in viscosity and a pool of liberated cell macromolecules. Subsequent extraction with phenol initiates deproteinization, destroying degrading enzymes and proteins. Centrifugation removes the cell debris and proteins from the aqueous layer (top) containing the nucleic acids to the phenol layer (bottom) and/or at the interface of the two layers.

Treatment with ethyl alcohol precipitates the nucleic acids from the aqueous layer and allows collection of the sample by spooling. Further purification with isopropanol will selectively precipitate the DNA from the RNA to provide a better yield of DNA product. Although the latter procedure has not been used here, it should be attempted to determine whether further purification is demonstrable.

Each sample is stored to be treated by acid hydrolysis. The resulting base hydrolysate are to be analyzed for purine and pyrimidine content as described under "Base Determinations."

Alternatively, extraction of DNA from various phages or mammalian cells should be attempted for comparisons of composition from various sources.

# BASE DETERMINATIONS

## Discussion, Results

The bacterial DNA samples obtained from the prior experiment by phenol extraction were hydrolyzed with formic acid and subjected to base composition analysis by paper chromatography. Analysis followed the general procedures described for this techniques, choosing the one-dimensional ascending technique at room temperature.

A set of controls were prepared to provide a basis for comparisons of separation and identification of components and correlation with known Rf values. Individual base controls were composed of known purine and pyrimidine bases prepared in HCL--adenine, guanine, thymine, cytosine, uracil, and 5-hydroxymethylcytosine. A mixture of these bases as well as a commercial grade of DNA and RNA, treated with acid hydrolysis in fashion similar to the bacterial DNA samples also were analyzed.

Several different sets of chromatograms were developed in the two solvent systems employed -- isopropanol-HCl and isopropanol-$NH_3$. All tests reflected varying problems. The first problem was insufficient resolution of the control bases (TABLE Ia). In many cases, guanine, adenine, and thymine failed to appear on the chromatograms in either solvent system. It is probable that the amount in solution or applied to the paper was insufficient. Measured quantities of each base must be used for controls. Solubility differences were observed. Thymine appeared most soluble. Both cytosine and 5-hydroxymethylcytosine were readily soluble. It is suggested that repeated application of all samples be made at the origin of each chromatogram. Cytosine was observable in both solvent systems with Rf values falling within the range of the values reported for the same system (TABLE I). It should be noted, however, that the values given were obtained by descending chromatography (3), but ascending values have been reported not to differ significantly, but they are frequently somewhat lower. We found 5-hydroxymethylcytosine was discernible only in the isopropanol-HCl solvent.

Similar problems frequently were encountered in the resolution of the control base mixture (TABLE II). Separation of only one component, cytosine, commonly was observable in the isopropanol-HCl system with correlation in position and value to the individual base control. A nucleic acid sample that has been extracted suitably and prepared free of extraneous contaminating substances should chromatograph with good separation and reflect a distinct base component profile, a profile representing relative purity of the original sample obtained. This was demonstrated for the analysis of the commercial DNA sample in the isopropanol-HCl system, with visible separation into four components and slightly higher Rf values. In the second solvent, resolution was less distinct and values relatively unsatisfactory for comparison purposes. The commercial RNA sample was poorly resolved and proved less useful. Unfortunately, the class bacterial DNA samples generally prove even less desirable for analysis. In the second experiment, resolution appeared as a continuous band profile with faint separation of possibly one component, identifiable by position and value as cytosine (TABLE III). The limited success may be attributable to an impure DNA sample, in which the extraction was poor, leaving contaminating substances which interfered with appropriate separation and resolution of components.

The thin-layer chromatography on silica gel plates has already been described as useful in the study of nucleic acid components. It is expensive and further study of procedures and development of appropriate materials will be needed to modify this method to class conditions.

Under the present experimental conditions, various problems have been encountered that limit successful analysis of nucleic acid samples. Paper chromatography has demonstrated utility as one method for analysis, especially as a means of determining composition and of relating purity of samples. Extension into the extraction of other DNA phages, RNA phages, mammalian viruses, or mammalian cell DNA should provide interesting comparisons for analysis of nucleic acids from a variety of these sources.

TABLE I. Rf Values of Purine and Pyrimidines*

| Base Component | Solvent | |
|---|---|---|
| | Isopropanol-HCl | Isopropanol-$NH_3$ |
| Adenine | 0.37 | 0.32 |
| Guanine | 0.16 | 0.22 |
| Cytosine | 0.22 | 0.44 |
| Thymine | 0.52 | 0.76 |
| Uracil | 0.38 | 0.66 |
| 5-Hydroxymethylcytosine | 0.25 | 0.44 |

* E. Chargaff and J. Davidson, The Nucleic Acids, Volume I, Chapt. 7, Academic Press, 1955.

TABLE Ia. Rf Values for Control Base Samples

| Base Component | Solvent System<br>Isopropanol-HCl | Isopropanol-$NH_3$ |
|---|---|---|
| Adenine | --- | --- |
| Guanine | --- | --- |
| Cytosine | 0.22 | 0.34 |
| | 0.29 | 0.40 |
| | 0.27 | 0.27 |
| | 0.28 | 0.23 |
| Thymine | --- | --- |
| 5-hydroxymethylcytosine | 0.14 | 0.08 |
| | 0.23 | --- |
| | 0.16 | --- |
| | 0.14 | --- |
| Uracil | not done | not done |

TABLE II. Rf Values for Control Mixtures

| Control | Component Identified | Solvent System<br>Isopropanol-HCl | <br>Isopropanol-$NH_3$ |
|---|---|---|---|
| Base Mixture | Cytosine | 0.23 | --- |
| | | 0.32 | --- |
| Commercial DNA | Adenine | 0.47 | 0.54 |
| | Guanine | 0.30 | --- |
| | Cytosine | 0.38 | 0.62 |
| | Thymine | 0.64 | 0.92 |
| Commercial RNA | Cytosine or Thymine | 0.34 | 0.44 |

TABLE III. Rf Values for Class Bacterial DNA (_E_. _coli_ B)

| Experimental Sample | Component Identified | Solvent System Isopropanol-HCl | Isopropanol-$NH_3$ |
|---|---|---|---|
| Sample 1a | --- | --- | --- |
| Sample 1b | --- | --- | --- |
| Sample 2a | Adenine | 0.372 | --- |
| Sample 2b | Cytosine | --- | 0.47 |

## ANIMAL VIRUSES: IN VITRO STUDIES

### Discussion, Results

### A. Preparation of Cell Monolayers -- Growth in Serum-free Media

Investigations have already been described which successfully employed serum-free medium for growth of a variety of cell lines in suspension cultures. An attempt is made here to evaluate the growth of cultures of chick cells in serum-free medium, especially in heat-stable, autoclavable peptone medium introduced by Nagle (5). Studies also were conducted with the more recently introduced heat-stable glutamine-free chemically-defined medium developed by Nagle and Brown (8). Cells were prepared as described in the laboratory procedures, suspended in serum-free medium and dispensed in glass tissue culture dishes. Observations of monolayer formation were made at 37 C incubation under $CO_2$ and normal incubation at 37 C, with consideration of attachment of cells to surface, pH changes, and appearance of cell sheets. Comparisons were made with control cells grown in serum-containing medium (Eagle's minimal essential medium).

Initial attempts to grow monolayer cultures proved unsuccessful, as cells remained rounded and failed to attach to the plate surface. It was possible, however, to obtain what appeared to be confluent cell monolayers under $CO_2$ for both types of media. Cell attachment was evident, and comparisons with control cultures indicate no significant differences in pH changes or appearance during early periods of growth. However, after 18-24 hours of growth, some difference in the completeness of the cell sheet can be discerned. There was a less pronounced flattening and elongation of cells grown in serum-free media.

Cell cultures grown and maintained in peptone medium in pyrex milk dilution bottles thus providing an airtight atmosphere were observed to grow with some success and form cell sheets similar in appearance to peptone plate cultures under $CO_2$ and with little pH change.

Studies were eventually extended to the growth and maintenance of continuous cell lines in serum-free media. HeLa and mouse L-cells gave little success upon initial transfer of cells directly into the medium. The cells would not attach and remained rounded. Each cell line was then slowly exposed to varying portions of serum-free and serum-containing media. Growth has been reported for suspension cultures, but it is apparent that a longer period of adaptation is required for monolayer cultures.

It has been noted that the serum-free medium employed appears to be nutritionally complete and adequate as chick cells are able to adapt and grow with some success. However, it should be considered that since the nutritional environment has been modified from the standard serum-containing media, e.g., a possible lack of additional growth factors, differences and alterations do exist. There may be qualitative differences in the formation and final appearance of monolayers in serum-free media. Addition of serum to the serum-free media produced cell sheets comparable to control cultures.

## B. Virus Growth and Plaque Assay on Serum-free Grown Monolayers

Serum-free cultures of chick cell monolayers were prepared as previously described and evaluated as to whether they could serve as effective systems for virus titrations. Plaque assays were conducted employing the large plaque variant of Sindbis virus. Plaque formation was investigated with respect to visibility of plaques, size, and numbers. Studies with the initial serum-free peptone cultures at one or two days of growth when monolayers seemed apparent indicated production of plaques, but results were variable, being influenced by the degree of confluence of the monolayers. When growth of monolayers in serum-free media appeared consistent, assays were carried out and comparisons made with assays on serum-containing control grown cultures (TABLE I and II). The plaques produced on the peptone-grown monolayers were visible, countable, but diffuse and less distinct as well as smaller than were the control culture plaques. In most cases, virus titers were comparable (TABLE I and II). Growth of cells and reading of assays may be facilitated on plastic plates, but no great difference was apparent (TABLE II).

Serum-containing cultures produced visible and prominent large plaques. With the addition of serum to the serum-free peptone overlays, it was possible to obtain plaque enhancement to a degree similar to control cultures. It also was noted that overlay of serum-free media on control cultures did not diminish visibility of plaques, but did increase plaque numbers (TABLE I).

Serum-free cultures of chick cell monolayers were investigated for their competence in supporting viral replication, as determined by monitoring the growth cycle of the large plaque variant of Sindbis virus during a 24-hour period. Parallel growth in serum-containing cultures were also monitored for comparisons and showed comparable results in virus growth rates and titers to those in serum-containing control cultures (GRAPH 1). It is also noted that storage of virus sample at 4 C for a short period indicated no greater decrease in original titer of the serum-free stock than for the control stock. A more pronounced decrease in virus yields was indicated after 10-12 hours of growth in the serum-free medium than in the control cultures (GRAPH 2).

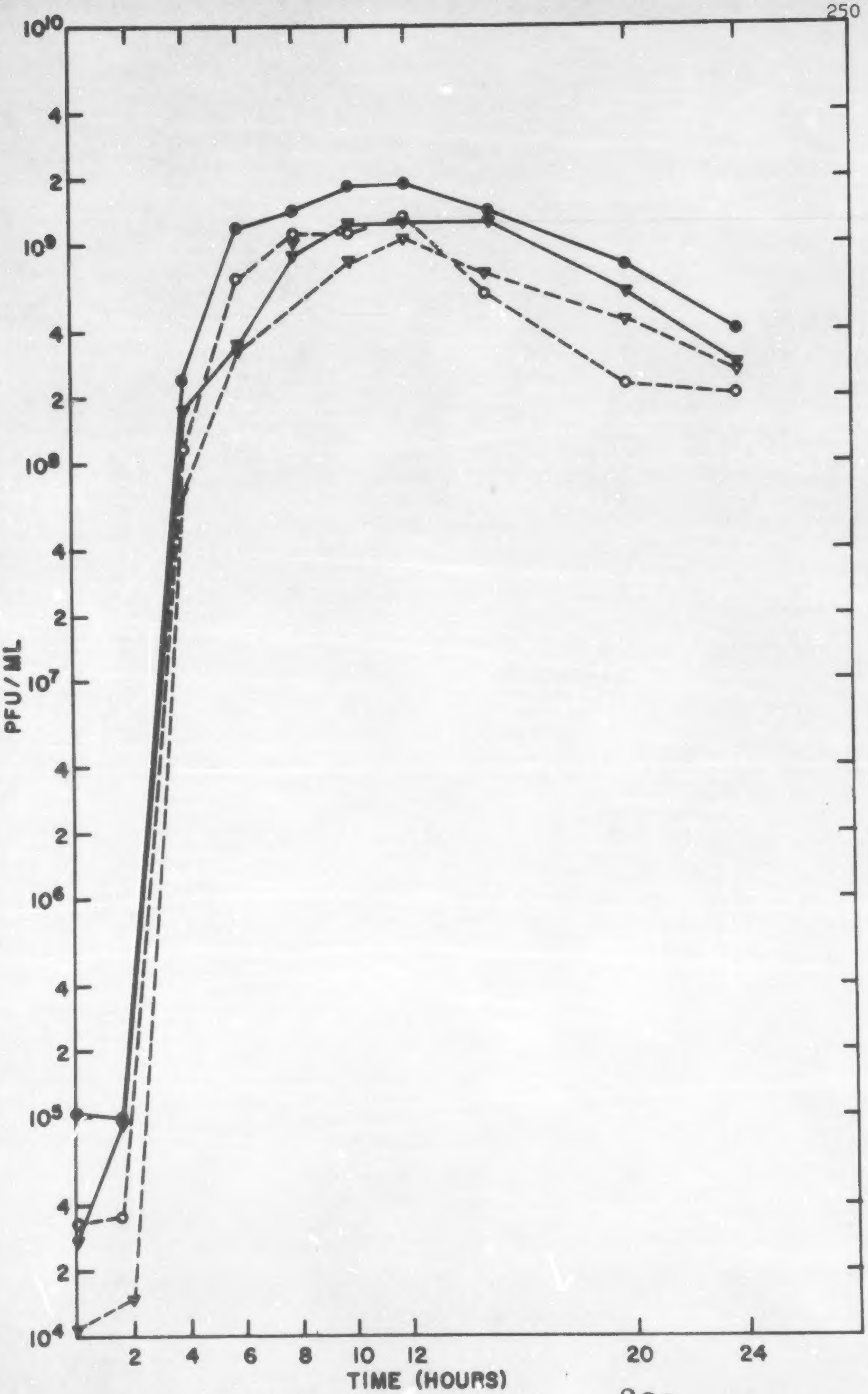
Presently, from the various studies described, serum-free cultures appear to provide a possible system for virus assay. Differing from conventional serum-containing cultures, the preparation of media involved fewer components and can be autoclaved easily rather than being sterilized by time-consuming filtration. Results indicate that the state of cell and consistency of monolayer growth are important in plaque formation. The observed higher efficiency of plating with serum-free medium reflect enhanced cell protection as well as non-specific virucidal activity.

Both Figures: Growth of LP Sindbis virus in serum-free and serum-containing media.

Figures 1-2.

Serum-free and serum-containing cultures of chick cell monolayers were prepared and infected with LP Sindbis virus at time zero. At indicated time intervals during a 24 hour growth period, duplicate samples of virus were harvested and later assayed for virus titers.

● serum-containing
○ serum-containing (stored)
▼ serum-free
▽ serum-free (stored)


10^10
4
2
10^9
4
2
10^8
4
2
10^7
4
2
10^6
4
2
10^5
4
2
10^4
PFU / ML
2
4
6
8
10
12
20
24
TIME (HOURS)

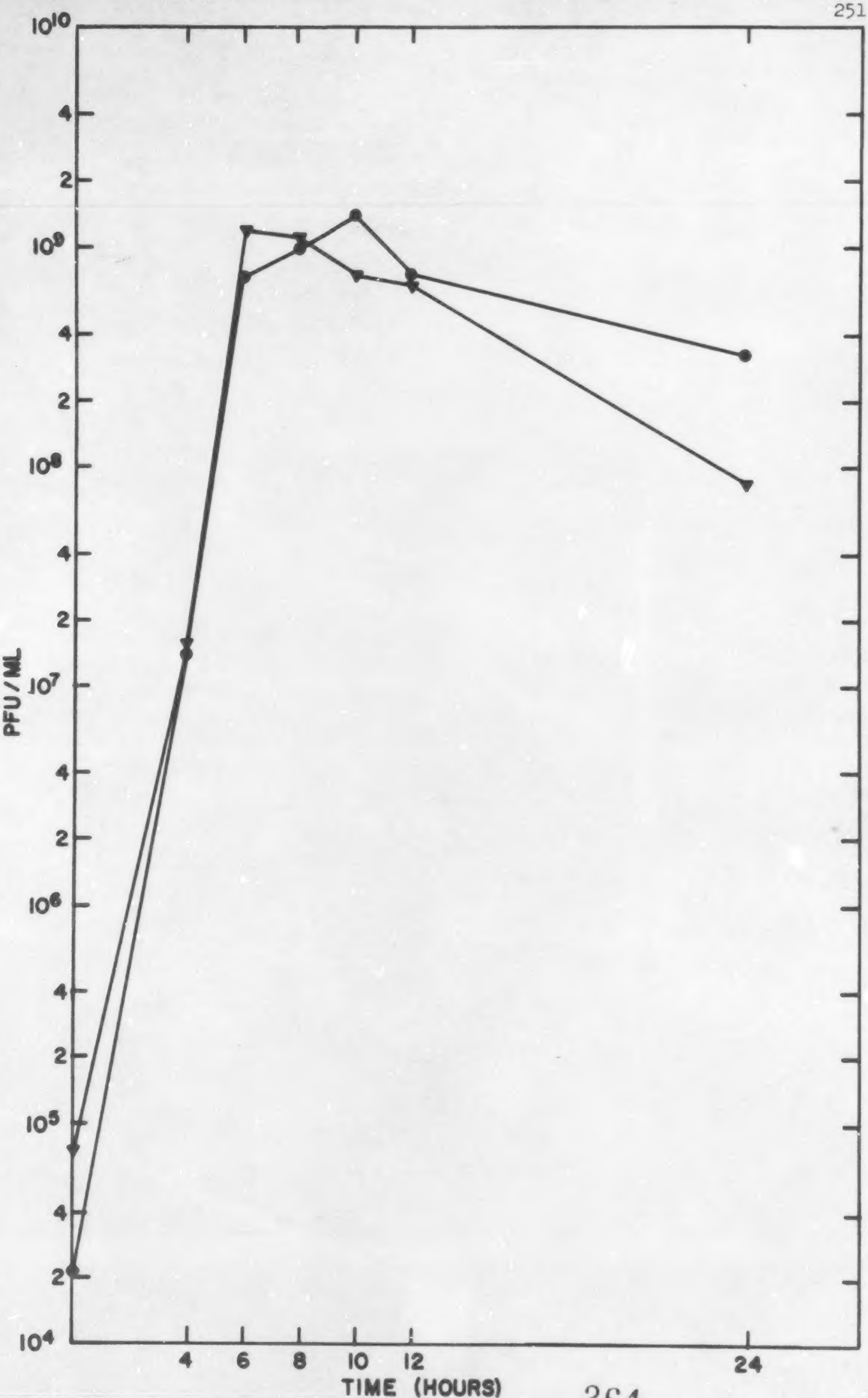

10^10
4
2
10^9
4
2
10^8
4
2
10^7
4
2
10^6
4
2
10^5
4
2
10^4
PFU/ML
4
6
8
10
12
24
TIME (HOURS)

TABLE I. Plaque Assay of Sindbis Virus (LP) on Serum-free (Peptone) and Serum-containing (Eagles) Monolayer Cultures

| Assay No. | Cultures | Type of Overlay--PFU per ml<br>Serum-free | Serum-containing |
|---|---|---|---|
| 1 | Pep-1 day | $1.0 \times 10^9$ | $7.0 \times 10^8$ |
| | Pep-2 day | $1.0 \times 10^9$ | $7.0 \times 10^8$ |
| 2 | Pep-2 day | $1.7 \times 10^9$ | $1.4 \times 10^9$ |
| 3 | Pep-2 day | $2.4 \times 10^9$ | $2.0 \times 10^9$ |
| 4 | Pep-1 day | $1.7 \times 10^8$ | $7.5 \times 10^7$ |
| 5 | Pep-1 day | $4.4 \times 10^7$ | $6.8 \times 10^7$ |
| 1 | Eagles-1 day | $9.6 \times 10^8$ | $8.5 \times 10^8$ |
| | Eagles-2 day | $1.1 \times 10^9$ | $5.4 \times 10^8$ |
| 2 | Eagles-2 day | $9.8 \times 10^8$ | $8.4 \times 10^8$ |
| 3 | Eagles-2 day | $9.0 \times 10^8$ | $8.4 \times 10^8$ |
| 4 | Eagles-2 day | $1.3 \times 10^9$ | $1.0 \times 10^8$ |
| 5 | Eagles-1 day | --- | $7.0 \times 10^8$ |
| | Glu-1 day | $1.0 \times 10^9$ | $8.8 \times 10^8$ |
| | Glu-2 day | $1.5 \times 10^9$ | $5.4 \times 10^8$ |

These results represent initial plaque assay studies.

Comparisons are made with the corresponding assay numbers indicated and the days indicate the age of the monolayers when employed for the assay.

Assay was conducted on glass tissue culture plates.

Virus adsorption was at 37 C for 30-60 minutes. Plaque readings made after 2 days of incubation at 37 C under $CO_2$.

TABLE II. Plaque Assay of Sindbis Virus (LP) on Serum-free (Peptone) and Serum-containing (Eagles) Monolayer Cultures

| Assay No. | Cultures | Types of Tissue Culture Plates<br>Plastic | Glass |
|---|---|---|---|
| 1 | Pep-1 day | $2.2 \times 10^9$ | $1.7 \times 10^9$ |
| 2 | Pep-1 day | $6.2 \times 10^9$ | $7.2 \times 10^8$ |
| 1 | Eagles-1 day | $9.0 \times 10^8$ | $7.5 \times 10^8$ |
| 2 | Eagles-1 day | $1.0 \times 10^9$ | $1.6 \times 10^9$ |

These results represent recent plaque assays performed on 60 mm plastic and glass tissue culture plates. Virus adsorption at 37 C for 30-60 minutes.

Plaque readings made after 2 days incubation at 37 C under $CO_2$.

## IN VIVO STUDIES

### Discussion, Results

Outbred conventional white mice, newborn and young adult weanlings, were inoculated with anesthesia intracerebrally and intraperitoneally with large plaque Sindbis virus containing about $10^4$ PFU per ml. Under these experimental conditions, mortality rates for newborns injected IC occurred between the 4th and 5th day, whereas newborns injected IP died between the 6th and 7th day. The onset of infection was manifested by several symptoms including decreased activity in movement and developing paralysis of the limbs with culmination in death. For weanlings injected IC and IP, however, both groups of mice remained well, showing no signs of apparent infection during a two week period.

Organs were collected 2 days and 5 days post-inoculation and prepared as homogenate suspensions for virus titrations. The results indicate that virus multiplication in both ages of mice was apparent but to a lesser degree in weanlings (TABLE I). The high viral titers in the brain tissue (a major target organ) and in the various organs of the newborn mice groups reflect widespread infection. A high viremia should be present as well, but the assay of the serum samples did not confirm this. This is time-dependent. The viral content in weanling organs showed limited multiplication, as indicated by the lower titers. Assays of serum samples were variable with presence of virus

2 days post-inoculation and no detectable virus after 5 days post-inoculation.

The data presented show that changes in susceptibility to Sindbis virus infection is age-dependent, as resistance develops with increasing age. Newborns lack a mature defense system and therefore are subject to infection which results in death whereas weanlings have developed resistance and are able to slow the progress of Sindbis infection.

Different studies have been cited (1) which have suggested that this resistance phenomenon may be attributable to a number of possibilities including development of some type of anatomical barriers in the resistant animals cells, a change in receptor site profile of the brain tissue of adult mice, an increased activity in the immune response, or resistance by maturation of the defense system as a function of age.

TABLE I. Recovery of LP Sindbis Virus from Various Organs and Tissues of Newborn and Weanling Mice

| Age of Mice (Days) | Day of Harvest p.i. | Route of Inoculation | PFU PER GRAM OF TISSUE | | |
|---|---|---|---|---|---|
| | | | Brain | Spleen | Liver |
| Newborn (2) | 2 days | IC | $3.7 \times 10^6$ | $2.1 \times 10^6$ | $1.5 \times 10^6$ |
| | | | $3.2 \times 10^6$ | $2.3 \times 10^6$ | $1.1 \times 10^6$ |
| | | IP | $3.5 \times 10^6$ | $2.3 \times 10^5$ | $8.6 \times 10^5$ |
| | | | $2.8 \times 10^6$ | $3.6 \times 10^4$ | $4.1 \times 10^6$ |
| Weanling (20) | 2 days | IC | $4.5 \times 10^5$ | $1.2 \times 10^3$ | $1.1 \times 10^5$ |
| | | IP | $9.6 \times 10^5$ | $1.5 \times 10^3$ | $2.1 \times 10^4$ |
| | 5 days | IC | $3.5 \times 10^4$ | $8.0 \times 10^1$ | $8.6 \times 10^1$ |
| | | IP | $3.2 \times 10^4$ | $1.4 \times 10^2$ | $2.9 \times 10^2$ |

Inoculum of 0.05 mls of Sindbis virus stock injected intracerebrally and intraperitoneally into both ages of mice.

# APPENDIX II

# MEDIA

# MEDIA

### Tryptose Phosphate Broth:

29.5 grams commercial tryptose phosphate broth powder

1000 mls distilled water

Autoclave and store at 4 C.

### Nutrient Broth:

8 grams Difco nutrient broth powder

5 grams NaCl

1000 mls distilled water; adjust to pH 7.4.

Autoclave and store at 4 C.

### Synthetic M9 Medium:

Prepare the following 3 sterile solutions:

(1) 40% glucose

40 grams glucose (dextrose)

100 mls distilled water

(2) salts in 10-fold concentrations:

10 grams $NH_4Cl$

30 grams $KH_2PO_4$

60 grams $Na_2HPO_4$

1000 mls distilled water

(3) $MgSO_4 \cdot 7H_2O$:

26.5 grams $MgSO_4 \cdot 7H_2O$

1000 mls distilled water

Combine the following amounts to prepare the stock solution:

75 mls glucose (to give 3% final concentration)

100 mls salts

10 mls $MgSO_4$

815 mls sterile distilled water

Mix and store at 4 C until use.

Diluent Broth:

Prepare nutrient broth as described above.

Prepare physiological saline:

9 grams NaCl

1000 mls distilled water

Autoclave.

Mix as 1 part broth to 4 parts saline. Keep chilled until use.

## AGAR MEDIA

### Soft Agar Overlay:

29.5 grams tryptose phosphate broth powder (May substitute nutrient broth powder for $TPO_4$.)

9.0 grams agar

1000 mls distilled water

Steam mixture to melt agar. Dispense as 2.5 ml or 7.5 ml volumes into tubes. Autoclave. Tubes should be stored at 4 C until use, when steamed to melt.

### Basal Agar Plates:

29.5 grams tryptose phosphate broth powder (May substitute nutrient broth powder for $TPO_4$.)

18.0 grams agar (1.8%)

1000 mls distilled water

Autoclave. Dispense as 18-20 mls per sterile petri dish.

### Agar Slants:

29.5 grams tryptose phosphate broth powder

1.5 grams agar (1.5%)

1000 mls distilled water

Steam to melt agar. Dispense as 5 mls per screw cap tube. Autoclave. Slant tubes and allow to solidify. Allow to cool at room temperature overnight. Check and discard contaminated slants. Store at 4 C until use.

Salt-free Agar Plates:

10-20 grams Bacto-tryptone

1 gram glucose

10 grams agar

1000 mls distilled water

Autoclave and adjust to 7.2. Dispense 15-20 mls per plate.

Salt-free Agar Overlay:

10 grams agar

1000 mls distilled water

Autoclave and dispense 2.5 mls per tube.

## HEAT-STABLE PEPTONE MEDIUM

1. Add the following components to distilled water:

| Component | Amount (mg/L) |
|---|---|
| Bacto-Peptone (Difco) | 1000 |
| Proteose Peptone (Difco) | 1000 |
| NaCl | 7400 |
| KCl | 400 |
| $CaCl_2 \cdot 2H_2O$ | 265 |
| $MgCl_2 \cdot 6H_2O$ | 275 |
| $NaH_2PO_4 \cdot H_2O$ | 100 |
| Glucose | 1000 |
| Na Pyruvate | 110 |
| L-cysteine · HCl | 75 |
| L-glutamine | 200 |
| Phenol Red | 15 |

2. Be sure all the components are dissolved. The solution may be dispensed into 100 ml serum bottles and autoclaved for 15 minutes. Allow the solution to cool and store at 4 C until use.

3. At the time of cell inoculation, add sufficient sodium bicarbonate (5%) to give the desired pH (cherry red color).

## HEAT-STABLE GLUTAMINE-FREE CHEMICALLY-DEFINE MEDIUM

1. Each of the following components is dissolved in the appropriate amount of hot distilled water (970 mls):

| Component | Amount (g/L) |
|---|---|
| L-alanine | 0.4 |
| L-arginine | 0.1 |
| L-asparagine | 0.3 |
| L-cysteine HCl | 0.075 |
| L-glutamic acid | 0.15 |
| L-histidine HCl | 0.06 |
| L-isoleucine | 0.15 |
| L-leucine | 0.3 |
| L-lysine | 0.3 |
| L-methionine | 0.06 |
| L-phenylalanine | 0.12 |
| L-proline | 0.3 |
| L-serine | 0.3 |
| L-threonine | 0.135 |
| L-tryptophan | 0.06 |
| L-tyrosine | 0.12 |
| L-valine | 0.15 |
| Glucose | 3.0 |
| Na pyruvate | 0.11 |

| Component | Amount (g/L) |
|---|---|
| NaCl | 7.4 |
| KCl | 0.4 |
| $CaCl_2 \cdot 2H_2O$ | 0.265 |
| Ferric $NH_4$ citrate | 0.003 |
| $MgCl_2 \cdot 6H_2O$ | 0.275 |
| $NaH_2PO_4 \cdot H_2O$ | 0.3 |
| $ZnSO_4 \cdot 7H_2O$ | 0.003 |
| Phenol Red | 0.01 |

2. Prepare the following vitamin solution as a 100× solution by adding the following ingredients to distilled water:

| Component | Amount (g/L) |
|---|---|
| D-biotin | 0.1 |
| Choline-Cl | 5.0 |
| Folic acid | 0.1 |
| Niacinamide | 0.1 |
| Ca Pantothenate | 0.2 |
| Pyridoxial · HCl | 0.1 |
| Thiamine · HCl | 0.1 |
| I-inositol | 0.1 |
| Riboflavin | 0.01 |
| $B_{12}$ | 0.002 |

The vitamin stock may be frozen and thawed for use. Add 10 mls of the 100× stock per liter of glutamine-free medium.

3. Autoclave the first mixture for 15 minutes. Allow to cool and dispense to 100 ml sterile serum bottles. When ready for use, add 5% sodium bicarbonate to obtain the suitable pH (cherry red color).

# HANKS' BALANCED SALT SOLUTION (10×)

| Component | Amount (g/L) |
|---|---|
| NaCl | 80.0 |
| KCl | 4.0 |
| $MgSO_4 \cdot 7H_2O$ | 1.0 |
| $MgCl_2 \cdot 6H_2O$ | 1.0 |
| $Na_2HPO_4 \cdot 2H_2O$ | 0.6 |
| $KH_2PO_4$ | 0.6 |
| Glucose | 10.0 |
| Phenol Red | 0.2 |
| *$CaCl_2$ | 1.4 |

Procedures:

1. Add components individually to 1/2 the final volume of distilled water. Allow each to dissolve.

*2. Prepare a separate solution of $CaCl_2$ and add as the last ingredient to the solution to give the sufficient concentration indicated. It is noted that $CaCl_2$ requires some time to dissolve in solution.

3. Bring up to final volume.

4. Correct the pH with saturated sodium bicarbonate solution (cherry red color).

5. Filter sterilize. Store at room temperature.

## PHOSPHATE BUFFERED SALINE (PBS) 10×

| Component | Amount (g/L) |
|---|---|
| NaCl | 80.0 |
| KCl | 2.0 |
| $Na_2HPO_4$ | 11.5 |
| $KH_2PO_4$ | 2.0 |
| $H_2O$ | Bring volume to 1 liter |

Procedures:

1. First dissolve $Na_2HPO_4$ in 200 mls of water.
2. Add 1/2 the final volume of water to the dissolved $Na_2HPO_4$.
3. Add the remaining components.
4. Bring up to the correct volume.

## TRYPSIN (0.125%)

Mix the following components:

1 vial of Difco trypsin

40 mls of 10× PBS

360 mls of sterile water

# APPENDIX III

## Aids for
## Individual Experiments

## PREPARATION OF BACTERIAL CULTURES

A. Indicator Cultures

1. Inoculate loopful of bacteria from stock slant culture into a volume of broth media in Erlenmeyer flask (medium volume should not exceed 1/5 flask capacity).
2. Incubate on shaker with vigorous action at 37 C for 18-24 hours.

B. Log Cultures (2.5 hours)

1. Prepare an 18-24 hour culture.
2. Make a 1:200 dilution of the overnight culture into fresh, prewarmed media.
3. Incubate the culture at 37 C for 2 to 2.5 hours, depending on the type of bacteria employed, the type and volume of medium used. For example, a log culture of *E. coli* β, grown in $TPO_4$, can be obtained within a 2 to 2.5 hour period, containing 3-5 × $10^8$ cells per ml.

C. Lysogenic Cultures

1. It has been observed that the growth of the experimental culture of *E. coli* K12 (λ) is facilitated in an enriched medium such as $TPO_4$ broth.
2. Inoculate from slant culture into the appropriate medium and incubate at 37 C 18-24 hours. Prepare a log culture by dilution of overnight culture into fresh, prewarmed medium (1:200). Incubate at 37 C for 2.5 hours.

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3. Centrifuge the culture at 2000 rpm in an International centrifuge for 20 minutes to pack cells. Resuspend the pellet in the same volume of synthetic M9 medium.
4. The culture can be exposed to UV irradiation. The source of UV employed was a GE germicidal lamp at a distance of 20 cm for 5 seconds.

## PREPARATION OF PHAGE STOCKS

### Broth Method

1. Prepare an overnight culture of E. coli β in one of several broths--tryptose phosphate broth, nutrient broth, or synthetic M9.
2. From the overnight culture, prepare a young culture by dilution (1:200) into fresh, prewarmed media. Any volume may be used. Incubate on a shaker at 37 C for 2-2.5 hours at which time the optical density of the culture at 550 mμ should have reached a reading of 0.5 to 0.6. For synthetic medium a longer lag period can be expected, approximately 3-4 hours.
3. Infect the culture with the desired phage at a M.O.I. of 1 phage per 50 cells. Aerate the mixture vigorously, or place on a shaker with vigorous action at 37 C for 5-6 hours.
4. At the end of the incubation period, add chloroform (1 ml per 20 mls of culture) and shake an additional 15 min at 37 C. Transfer the contents to a sterile centrifuge tube or bottle and centrifuge at 6000 rpm for 20-30 min to sediment cellular debris. Save the supernatant as phage lysate. Add a small amount of chloroform to the lysate to help maintain sterility, shaking vigorously for a few minutes. Allow to settle.
5. Titrate the phage stock, storing at 4 C.
6. This method allows for the preparation of several hundred milliliters of lysate with a phage yield of 1-5 × $10^{10}$ virus particles per milliliter.

## Plate Method

1. Determine the near confluent lysis titer of the phage stock by the conventional soft agar overlay technique.
2. Prepare a 12-15 hour culture of E. coli β. Inoculate 30 basal agar plates using 0.1 ml of the indicator culture and 0.1 ml of the near confluent lysis dilution of the phage into each of 2.5 ml of soft agar overlay.
3. Overlay and allow to solidify. Incubate all plates at 37 C for 12-15 hours.
4. Harvest the plates by addition of 3 mls of warm medium per plate. Allow the plates to sit for 15-20 minutes.
5. Gently scrape off the soft agar layer with a sterile glass L-rod into a sterile Ten Broeck tissue homogenizer. Homogenize with three to four pestle strokes. Decant homogenate into sterile centrifuge tubes or bottles. Complete all thirty plates in a similar manner. Rinse homogenizer with 5-15 mls of additional medium and decant into bottle.
6. Add chloroform to the pooled homogenates (1 ml per 20 mls of culture). Shake vigorously. Centrifuge at 7500 rpm for 10-15 minutes. Decant and save the supernatant as phage lysate. Additional chloroform may be added to maintain sterility. Titrate and store at 4 C.
7. This method allows preparation of about 100 mls of phage lysate with a yield of $10^9$-$10^{10}$ phage particles per milliliter. Alternatively, the soft agar layer may be homogenated through a large syringe (30 or 50 ml), or scraped directly into a centrifuge bottle with no

homogenization. Add the chloroform; shake vigorously and centrifuge. Phage yields are comparable.

# PREPARATION OF PHAGE ANTISERA

## A. Immunization

Materials:

sterile syringe

70% ethanol

cotton or gauze pads

2 rabbits for each phage stocks

designated phage stocks ($10^{10}$ to $10^{11}$ PFU per ml)

Procedure:

1. Centrifuge crude phage lysates several times at a moderate speed (e.g., 6000 rpm for 15-20 min) to remove bacteria and debris before use.
2. Several routes of injection may be used -- subcutaneous, intravenous, or intraperitoneal. Subcutaneous is the more simple method.
3. Sterilize the skin area by rubbing with 70% ethanol. Before injection, check syringe to avoid air embolism. Tap lightly to dislodge any trapped air bubbles.
4. Two 1 ml injections per week over a three week period appear to be adequate. A booster injection will increase the final serum titer if given about two weeks after the end of the first course.

## B. Collection of Antisera

Materials:

inoculated rabbits

Goose neck lamp or xylene

soap and water

sterile centrifuge tubes -- 30 or 40 ml

gauze or cotton

razor blades

Procedures:

1. The animals are usually bled one week after the last injection.
2. Carefully shave over the inner marginal vein of the right ear with the razor blade.
3. Dilate the vein with a heating lamp or rub with xylene.
4. With a quick stroke from the razor, make a slit across the marginal ear vein sufficient to nick but not sever the vein. Hold the top of the ear in a horizontal position and allow the blood to drip into a centrifuge tube. (Collect an initial test sample of 2-3 mls if desired).
5. If xylene is used, wash the area with soap and water before placing a piece of cotton over the cut. Press firmly and remove when bleeding has ceased.
6. Allow the blood to clot at 37 C and carefully separate the clot from the tube wall. Place in the refrigerator overnight.
7. Centrifuge at 1000 g (2500 rpm) in an International centrifuge for 15-20 min.

8. Remove the serum with a sterile Pasteur pipette. Repeat centrifugation to remove residual red blood cells or bits of clot.
9. Assay the test sample for serum potency. If adequate, rabbit may be bled again, collecting as much as 40-50 mls of blood every two-three days for two weeks using the procedure described.
10. After a week's rest, the rabbit may be given a second set of injections and bled again. All serum samples can be stored in screw cap vials or bottles and preserved by freezing at -20 C. It is desirable to freeze in small amounts.

## C. Testing Potency of Antisera

Materials:

homologous and/or heterologous phage stocks (diluted to a level of 1-5 × $10^7$ PFU per ml)
antisera samples
basal agar plates
soft agar overlays
sterile dilution tubes
chilled diluent
sterile 1.0 ml pipette
sterile 10 ml pipette
ice bath

Procedures:

1. Dilute the test antisera sample to various concentrations, e.g., 3-fold, 10-fold, and so forth.

2. All samples and materials should be prewarmed at 37 C for 10-15 min to avoid temperature shifts during the experiment.
3. At time 0 transfer 0.1 ml of each test antisera dilution and mix with a 0.9 ml phage sample. Immediately make the appropriate dilutions. Inoculate 0.1 ml of the selected dilutions to seeded soft agar overlays and plate.
4. Repeat procedures at 5' and 30' for each antisera concentration.
5. Incubate all plates 18-24 hours at 37 C.

The dilution of antiserum that yields 50-250 PFU per ml is the most useful for neutralization tests.

A more approximate and precise value for serum potency may be determined from the equation:

$$K = 2.3D/t \log B/P$$

# PREPARATIONS FOR T4 MORPHOGENESIS

## A. Preparation of T4 Amber Mutants

1. Prepare an overnight culture of the permissive host *E. coli* CR63 in broth. Incubate at 30 C on shaker.
2. Prepare a log phase culture of *E. coli* CR63 by dilution of the overnight culture 1:200 into fresh medium. Incubate at 30 C for 2 - 2.5 hours with an approximate optical density reading of 0.5.
3. Infect with the desired amber mutant (T4 am N52, B252, B17, or X4E) at a m.o.i. of 0.01 PFU per cell. Shake while incubating at 30 C for 5-6 hours.
4. Add chloroform (1 ml per 20 mls of culture) to lyse the cells. Shake for 15-20 minutes.
5. Centrifuge at 6000 × g for 20 minutes.
6. Decant and save supernatant as phage lysate. Phage stocks are titered using the permissive host *E. coli* CR63. Preparation of phage stocks under these conditions for 100 mls of culture will give a phage titer of $10^8$-$10^9$ PFU per ml.

## B. Preparation of Infected-cell Extracts

1. Prepare an overnight culture of the non-permissive host *E. coli* β. Incubate at 37 C on shaker.
2. Prepare a log phase culture of *E. coli* β by dilution of the overnight culture 1:200 into fresh medium. Incubate at 37 C for 2.5 hours to obtain approximately 5-7 × $10^8$ cells per ml. Prepare 25 mls of culture for each student assay extract.

3. Cool the culture to 30 C at room temperature or in an ice bath.
4. Infect the culture with the desired mutant (amN52, B252, or B17) at a m.o.i. of 4 PFU per cell. Aerate vigorously at 30 C for 30 minutes.
5. Cool the culture in an ice bath for 5-10 minutes. Centrifuge at 5000 × g for 10 minutes in Sorvall refrigerated centrifuge.
6. Resuspend the cell pellet in 2 mls of buffer containing DNAse at 10 μg/ml for each 25 mls of culture prepared. The cell pellet will be very viscous and will require pipetting to obtain adequate dispersion.
7. Transfer to a screw-capped tube. Freeze the pellet in a dry ice-ethanol bath. Thaw at room temperature.
8. The cell extracts may be used after thawing, or stored by refreezing in the dry ice-ethanol bath and kept at -20 C until use.

## C. Preparation of Tail-Fiberless Particles

1. Prepare an overnight culture of E. coli β at 37 C.
2. Prepare a log phase culture of E. coli β by dilution of the overnight culture 1:200 in fresh medium. Incubate at 37 C for 2.5 hours to obtain about 5-7 × $10^8$ cells per ml. Prepare 25 mls of culture for each student assay extract.
3. Infect with the mutant amX4E at a m.o.i. of 4 PFU/cell. Aerate the culture for 3 hours at 30 C. Lyse with chloroform (1 ml per 20 mls of culture). Shake for an additional 10-15 minutes.

4. Centrifuge at 5000 × g for 10 minutes to remove cell debris. Save the supernatant and centrifuge in the ultracentrifuge at 35,000 × g for 1 hour to sediment the tail-fiberless particles.
5. Decant and resuspend the pellet with 2 mls of buffer containing DNAse (10 μg/ml). Obtain an optical density reading of 1.0 at 265 mμ. Assume a phage particle concentration of about 1.0 - 1.2 × $10^{11}$ particles per ml.

D. Preparation of Reagents

1. Buffer

   Prepare 1 liter of buffer with the following ingredient concentrations:

   0.039 M $Na_2HPO_4$
   0.022 M $KH_2PO_4$
   0.07 M NaCl
   0.01 M $MgSO_4$

   Adjust to pH 7.4. Add DNAse at a concentration of 10 μg/ml.

2. Deoxyribonuclease (DNAse)

   (Bovine, grade 3, obtained from Miles-Seravac (Pty) Ltd.) with the following data:

   Activity 390 μ/mg
   Salt-free
   Absorption E280 = 12.5
   Lyophilized, stored below 0 C

   For use, add the desired amount to buffer.

## PREPARATIONS FOR ENZYME INDUCTION

A. Preparation of Substrates

1. lactose and glycerol

   These inducers are used as a 0.5% solution (weight/volume) in synthetic medium containing no glucose.

2. methyl-β-D-thiogalactoside (Sigma Chemical Company, St. Louis)

   This reagent is used as a 0.004 M (M.W. 210.3) solution in synthetic medium containing no glucose.

B. Preparation of Reagents

1. N-methyl-N'-nitro-N-nitrosoguanidine (Sigma Chemical Company, St. Louis)

CAUTION: This is a carcinogenic and mutagenic agent. Avoid skin contact.

   Dissolve the nitrosoguanidine in sterile water or directly in the appropriate media at a concentration of 20 μg/ml (M.W. 147.1). It should be noted that nitrosoguanidine does not go into solution readily. Frequent agitation of the mixture is required to aid in solution.

2. O-nitrophenylgalactoside (Sigma Chemical Company, St. Louis).

   This color indicator is used as a 0.013 M (Anhydrous, M. W. 301.3) solution in 0.01 M phosphate buffer at pH 7.0, just prior to use. Prepare a 0.2 M stock phosphate buffer containing monobasic phosphate ($NaH_2PO_4 \cdot H_2O$) and dibasic phosphate ($NaHPO_4$) in distilled water. To obtain pH 7.0, mix the solutions in the following proportions:

39 mls of 0.2 M $NaH_2PO_4 \cdot H_2O$ + 61 mls of 0.2 M $NaHPO_4$

For use, dilute 1:20 to obtain a 0.01 M phosphate buffer.

# PREPARATIONS FOR DNA EXTRACTION

## A. Preparation of Bacterial Cell Mass

Prepare 100-200 mls of a 15-18 hour E. coli β culture grown in tryptose phosphate or nutrient broth at 37 C. Centrifuge the culture at 6500 rpm for 15-20 minutes. Wash the pellet with saline-citrate buffer and recentrifuge. This method should provide sufficient cell mass for one DNA extraction (approximately 1 ml packed cells).

If a large number of cell pellets are required, the batch method may be employed. A large vat containing 14-16 liters of medium is inoculated with 100 mls of a 24 hour culture of E. coli β and incubated at 37 C. Bubbling air through the culture to increase oxygen tension will aid in increasing the rate of bacterial cell growth. Centrifuge the culture and pool all cells. Wash with saline-citrate buffer and centrifuge again. Determine the cell mass needed and resuspend the centrifuged culture in an appropriate amount of saline-citrate buffer. Distribute as 1 ml sample for each student extraction.

If necessary, the cell pellets may be stored at 4 C until use.

## B. Preparation of Reagents

### 1. Lysing Medium

Prepare the following solutions just prior to use:

1 M Tris at pH 8.0

1.2 M EDTA (ethylenediaminetetraacetic acid)

Egg white lysozyme, 4 mg/ml

Combine in the following proportions:

100 mls 1 M Tris

4.5 mls 1.2 M EDTA

2.5 mls lysozyme

For use, dilute the prepared solution 1:20.

2. Saturated Phenol

For effective results, the phenol should be redistilled to remove contaminating ions. Prepare as a water-saturated, neutralized phenol solution (80%) and store in dark bottles at 4 C until use.

3. Saline-citrate Buffer

Prepare a 0.15 M NaCl solution. Add sodium citrate to the salt solution to give a final concentration of 0.015 M of sodium citrate. Adjust to pH 7.1.

4. SDS Detergent

Prepare as a 1.5% detergent by addition of sodium dodecyl or lauryl sulfate to saline-citrate buffer. Adjust to pH 7.1.

## PREPARATIONS FOR BASE DETERMINATIONS

### A. Preparation of Base Hydrolysates

1. One day prior to use in the laboratory, transfer the spooled DNA sample to a lyophilizing vial containing 0.5 ml of 88% formic acid. Seal the tip with a hot flame.
2. Autoclave at 121 C (15 lbs pressure) for 1 hour.
3. Carefully break the tip of the vial and evaporate in an 80 C water bath.

   NOTE: It has been observed that a long evaporation period (4-5 hours) is required. It may be shortened by directing air from a fan blower over the vial to aid in circulating acid fumes.
4. After evaporation, the samples are redissolved with 0.1 ml of 0.1 N HCl.
5. The sample is covered and stored at 4 C until use for analysis the following day.

### B. Preparation of Base Eluates

1. Base discs should be cut out from the paper chromatograms and shredded into smaller pieces. Transfer to clean test tubes.
2. Several hours prior to the laboratory (4-6 hours), add 5 mls of 0.1 N HCl to each test tube to immerse the shredded samples.
3. Place on a shaker for continuous agitation during the extraction period.

4. The eluted samples are presented to students for centrifugation and preparation for optical density determinations.

C. Preparation of Developing Solvents

1. Isopropanol-HCl System

   Mix 65 mls of reagent isopropanol with 16.7 mls of 12 N HCl. Bring to a total volume of 100 mls with deionized water.

2. Isopropanol-$NH_3$ System

   Mix 85 mls of reagent isopropanol with 1.3 mls of $NH_4OH$ (28% $NH_3$). Add 15 mls of deionized water.

D. Preparation of Individual Base Controls

1. A quantity of each base should be measured and dissolved. The solvents employed have been reported to resolve up to 75 μg per spot of each base. It is suggested that 100 μg of base be dissolved in 0.1 ml HCl.

2. Apply 0.05 ml of the solution to each chromatogram. Trial runs should be tested to determine whether this amount gives satisfactory resolution. If not make appropriate modifications in the sample amounts; repeated spotting can be used.

E. Preparation of Base Control Mixtures

1. Dissolve the determined amount (from above) of each base into 0.1 ml HCl.

2. Subject commercially prepared DNA to acid hydrolysis with 88% formic acid as described in section A, Preparation of Base Hydrolysates.

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